

FORM PTÖ-1390

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NUMBER

BTGI-0025

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (if known see 37 C.F.R. 1.5)

10/088830

INTERNATIONAL APPLICATION NO.
PCT/EP00/09325

INTERNATIONAL FILING DATE
25 September 2000 (25.09.2000)

PRIORITY DATE CLAIMED
24 September 1999 (24.09.1999)
11 November 1999 (11.11.1999)

TITLE OF INVENTION

WHEAT DP PROTEINS AND USES THEREOF

APPLICANT(S) FOR DO/EO/US

Crisanto GUTIERREZ-ARMENTA; Elena RAMIREZ-PARRA, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. X This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. X A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. X has been transmitted by the International Bureau.
 - c. is not required, as the application was filed in the United States Receiving Office (RO/US)
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. X Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. have been transmitted by the International Bureau.
 - c. have not been made; however, the time limit for making such amendments has NOT expired.
 - d. X have not been made and will not be made.
8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. X An oath or declaration of the inventor(s) 35 U.S.C. 371(c)(4).
10. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
- 1) Copy of the Application as published by WIPO under WO 01/21644, including the International Search Report
 - 2) Copy of the International Preliminary Examination Report
 - 3) Sequence Listing in written form (11 sheets) and computer readable format (1 Diskette)
 - 4) Transmittal Letter submitting sequence listing (1 sheet)
 - 5) Statement to support filing and submission of sequence listing (1 sheet)
 - 6) Return Receipt postcard

EXPRESS MAIL Mailing Label No. EV 069154253

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Date of Deposit: 22 March 2002

REGISTRATION NUMBER 38,525 and 38,534 Respectively

10010708883002

JC10 Rec'd PCT/PTO 22 MAR 2002

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#2/a

In re application of:

Gutierrez-Armenta and Ramirez-Parra

International Application No.: **PCT/EP00/09325**

Filed: **25 September 2000**

For: **Wheat DP Proteins And Uses Thereof**

EXPRESS MAIL INFORMATION

EXPRESS MAIL LABEL NO: EV 069154253 US

DATE OF DEPOSIT: March 22, 2002

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

PRELIMINARY AMENDMENT

The present Preliminary Amendment is filed along with the U.S. national phase application for International Application No. PCT/EP00/09325. Prior to examination on the merits, Applicants respectfully request that the application be amended as follows.

In the Specification:

Please insert the following paragraph after the title on page 1:

--CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is the U.S. national phase of International Application No. PCT/EP00/09325 filed September 25, 2000, which claims priority to Application No. ES 9902474 filed November 11, 1999 and to Application No. ES 9902127 filed September 24, 1999, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION--

JC10 Rec'd PCT/PTO 22 MAR 2002

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PATENT

Please insert the following header between lines 8 and 9 on page 1:

--BACKGROUND OF THE INVENTION--

Please insert the following header between lines 10 and 11 on page 5:

--SUMMARY OF THE INVENTION--

Please insert the following paragraph between lines 27 and 28 on page 5:

--BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequence of TmDP (wheat DP) (SEQ ID No 1) and the amino acid sequence encoded thereby (SEQ ID No 2).

Figure 2 shows an alignment of TmDP protein amino acid sequence with available sequences of DP proteins of animal origin (the asterisk * indicates amino acid identity among all sequences).

Figure 3 shows an alignment of partial sequences from the TmDP sequence and a deduced amino acid sequence corresponding to an *Arabidopsis thaliana* genomic DNA sequence of previously unknown function.--

Please replace the paragraph beginning at page 7, line 11 of the specification with the following rewritten paragraph:

--Algorithms and software suitable for use in aligning amino acid or nucleotide sequences for comparison and calculation of sequence homology or identity will be known to those skilled in the art. Significant examples of such tools are the Pearson and Lipman search based FAST and BLAST programs. Details of these may be found in Altschul et al (1990), J. Mol. Biol. 215: 403-10; Lipman D J and Pearson W R (1985) Science 227, p1435-41. Publicly available details of BLAST may be found on the internet at www.ncbi.nlm.nih.gov/BLAST/blast-help.html. Thus such homology and identity percentages can be ascertained using commercially or publicly available software packages incorporating, for example, FASTA and BLASTn software or by computer servers on the internet. Examples of the former are the GCG program package (Devereux et al Nucleic Acids

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Research (1984) 12 (1): 387) and the Bestfit program (Wisconsin Sequence Analysis Package, eg. Version 8 for Unix or IBM equivalent, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) which uses the local homology algorithm of Smith and Waterman, Advances in Mathematics, 2:482-489 (1981). Many international institutes, eg. Genbank (see www.ncbi.nlm.nih.gov/BLAST) and EMBL: (see, www.embl-heidelberg.de/Blast2), offer internet services.--

In the Claims:

Please amend claims 1-47 to read as follows:

1. (Amended) A method of controlling one or more of plant growth, gene expression, cellular DNA replication, cell cycle progression, differentiation and development comprising increasing or decreasing E2F-dimerization partner (DP) protein activity in a plant cell through expression of a recombinant DP peptide or protein in the plant cell, wherein the peptide comprises SEQ ID No. 2, a functional part thereof, or a sequence having at least 70% homology to either, wherein the peptide or protein is capable of interacting with a plant E2F protein or peptide to alter E2F activity in the plant cell.
2. (Amended) The method of Claim 1 wherein the peptide or protein sequence is of 50% or more identity with that of the corresponding full length or part of SEQ ID No. 2.
3. (Amended) The method of Claim 1 wherein the peptide or protein sequence is of 70% or more identity with that of the corresponding full length or part of SEQ ID No. 2.
4. (Amended) The method of Claim 1 wherein the plant DP activity comprises one or both of (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate E2F binding to E2F/DP transcription factor binding sites in plant DNA.

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5. (Amended) The method of Claim 1 further comprising the steps of altering the plant DP protein level, the E2F-DP DNA-binding activity, transactivation properties, and/or DP/E2F-binding activity.
6. (Amended) The method of Claim 1 wherein the DP may be modified alone and/or in combination with a modification of the levels or activity of plant E2F and/or plant Rb.
7. (Amended) An isolated, enriched, cell free and/or recombinantly produced protein or peptide, capable of altering E2F-dimerization partner (DP) activity in a plant cell comprising one or both DP activities in plants selected from (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate E2F binding to E2F transcription factor binding sites in plant DNA or effect thereof, wherein the protein or peptide comprises SEQ ID No. 2 or a functionally active part thereof or a sequence having at least 70% homology to such sequence or part.
8. (Amended) The protein or peptide of Claim 7 comprising at least 50% identity to SEQ ID No 2 or a functional active part thereof.
9. (Amended) The protein or peptide of Claim 7 comprising SEQ ID No 2 or a variant thereof modified such that the amino acid sequence is mutated such that its ability to dimerize with E2F protein is reduced from that of the native sequence or abolished completely therefrom, whereby the peptide is capable of acting as a DP protein which decreases or abolishes native or recombinant E2F binding to its DNA binding site, thus inhibiting or abolishing E2F activity in a cell in which it is present.
10. (Amended) The protein or peptide of Claim 7 comprising a sequence found in SEQ ID No 2 or having at least 70% homology thereto selected from the group consisting of SEQ ID No 6 ARAAMAPPRGGAAAAATAALDLTG VHILEAS SVPPLPERGGNAVQRKGAVDP, SEQ ID No 8 DKDRKKEKAAAPRITGWGLREYSKIVCEKVEAKGRT TYNEVADEIYSELKS, SEQ ID No

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10 MAHIGQGFDEKNIRRRVYDAFNVLIALRVIAKEKKEIR WMGLSNRYYEKIKKLEEV, SEQ ID No 12 RKELV NKIRNKKALLQEIEKQFDDLQNIKLRNQTLESS AENVNGIRLPFVLVKTSR, and SEQ ID No 14 KARVEIEISDDSKFAHFENGAPFTLHDDLSILEGVRGNS IGKAGRATLH.

11. (Amended) The protein or peptide of Claim 10 wherein the sequence comprises two or more of SEQ ID No 6, SEQ ID No 8, SEQ ID No 10, SEQ ID No 12, and SEQ ID No 14 or sequences at least 70% homologous thereto.

12. (Amended) An isolated, enriched, cell free and/or recombinant nucleic acid comprising a sequence encoding for expression of a protein or peptide of Claim 7.

13. (Amended) The nucleic acid of Claim 12 comprising the coding nucleic acid sequence of SEQ ID No 1 or a part thereof encoding for all or a functional part of the amino acid sequence shown therein.

14. (Amended) The nucleic acid of Claim 12 wherein the nucleic acid is contained in plasmid pCLON33, deposit number CECT 5195 made on August 17, 1999.

15. (Amended) The nucleic acid of Claim 12 wherein the nucleic acid encodes a plant DP or a functional variant thereof and comprises SEQ ID No 1, a sequence complementary to SEQ ID No 1, or a sequence that is antisense to SEQ ID No 1.

16. (Amended) A nucleic acid probe comprising a DNA sequence which encodes an amino acid sequence selected from the group consisting of SEQ ID No 2, SEQ ID No 4, SEQ ID No 6, and SEQ ID No 8.

17. (Amended) A nucleic acid probe or primer comprising a double or single stranded DNA sequence comprising 10 or more contiguous nucleotides of SEQ ID No 1 with the proviso that the

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ten or more contiguous nucleotides are not selected from nucleotides encoding amino acids 70 to 136.

18. (Amended) An oligonucleotides probe comprising at least 18 contiguous bases of SEQ ID No. 1.
19. (Amended) The oligonucleotide probe of Claim 18 comprising 30 to 100 bases.
20. (Amended) The oligonucleotide primer of Claim 17 comprising 10 to 20 bases.
21. (Amended) A DNA which is antisense to a nucleic acid of claim 12.
22. (Amended) A nucleic acid encoding the DP protein or peptide encoding sequence of Claim 7 with a sequence encoding an E2F protein or peptide.
23. (Amended) The nucleic acid of Claim 22 wherein the sequences encoding the DP and E2F are under control of the same regulatory element or elements.
24. (Amended) A nucleic acid vector or construct comprising a nucleic acid of Claim 12 or antisense nucleic acid thereto.
25. (Amended) A plant cell comprising recombinant nucleic acid, a vector or a construct of Claim 12.
26. (Amended) A transgenic plant or part thereof comprising recombinant nucleic acid, a vector, DNA construct or cell of Claim 12.
27. (Amended) An antibody against a DP peptide or protein of Claim 7.

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28. (Amended) The antibody of Claim 27 which is capable of specifically binding with plant DP factor peptides or proteins of Claim 7, but not to the human, mouse or *Xenopus* DP.

29. (Amended) A method for identifying and/or isolating DNAs corresponding to complete or partial genes that are regulated in G1 passage, G1/S phase transition and/or S phase progression of the cell cycle comprising contacting a sample of genomic DNA with a binding material specific for binding such complete or partial genes, removing non-bound DNA from the specific material, and releasing and isolating the bound DNA;

wherein the specific binding material comprises a peptide or protein comprising the DNA binding sequence of a protein that is capable of acting as a part of a plant hetero-oligomer transcription activator or repressor.

30. (Amended) The method of Claim 29 wherein the specific binding material comprises a peptide or protein which comprises a plant E2F DNA binding domain.

31. (Amended) The method of Claim 30 wherein the specific binding material comprises a peptide or protein which comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) hetero-dimerization domain and/or a plant retinoblastoma protein E2F binding domain.

32. (Amended) The method of Claim 29 wherein the material comprises a peptide or protein that comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) binding domain together with a peptide or protein that comprises a plant dimerization partner (DP) E2F binding domain.

33. (Amended) The method of Claim 29 wherein the E2F and DP domains are comprised in *Triticum monnoccoccum* sequences or functional variants or parts thereof.

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34. (Amended) The method of Claim 33 wherein the DP domains are comprised in peptides or proteins of claim 7.
35. (Amended) The method of Claim 29 wherein the specific binding material peptide or protein is labeled or tagged to assist in identifying immobilizing it when in bound complex with the gene to be identified and/or isolated.
36. (Amended) The method of Claim 29 wherein the specific binding material comprises two peptides or proteins, one comprising the E2F DNA binding domain, and one comprising the DP hetero-dimerization domain bound together as a hetero-dimer.
37. (Amended) The method of Claim 29 wherein the E2F sequence is all or part of SEQ ID No 4 or a sequence having 90% homology thereto.
38. (Amended) The method of Claim 29 wherein the binding material comprises a peptide or protein including an optionally labeled plant retinoblastoma protein E2F binding domain.
39. (Amended) A specific binding material comprising a peptide or protein having DNA binding activity with respect to plant DNA transcription activator or repressor factor binding sites and having the ability to dimerize or oligomerize with a further such plant protein together with one or more of said further peptides or proteins.
40. (Amended) A specific binding material comprising
- (i) a peptide or protein having DNA binding activity with respect to plant DNA E2F transcription factor binding sites and having the ability to dimerize with plant DP protein together with one or both of
 - (ii) a peptide or protein that is capable of binding to plant E2F through its DP hetero-dimerization domain, and

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(iii) a peptide or protein that is capable of binding to plant E2F through its retinoblastoma protein binding domain.

41. (Amended) The material of Claim 40 wherein the peptides or proteins are wheat peptides or proteins, functional variants thereof, or parts thereof.

42. (Amended) The material of Claim 40 comprising a hetero-dimer of (i) and (ii) or (i) and (iii).

43. (Amended) The material of claim 40 comprising both of the peptides or proteins (i) and (ii) in the form of a hetero-dimer together with peptide or protein (iii).

44. (Amended) The material of Claim 40 wherein the peptides or proteins are in labeled or tagged form.

45. (Amended) The material of Claim 40 wherein it is in the form of a coating or otherwise bound form on a support material.

46. (Amended) The material of Claim 40 wherein the material is in the form of particles of the peptides or proteins and/or hetero-dimers or trimers.

47. (Amended) A nucleic acid encoding a peptide or protein of Claim 7 fused to a sequence encoding a protein label.

REMARKS

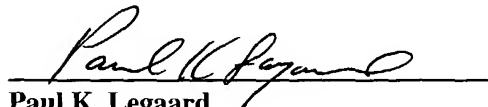
Claims 1-47 were originally filed in International Application No. PCT/EP00/09325. Claims 1-47 have been amended solely to place the claims in condition for national phase entry in the United States. No new matter has been added. Upon entry of the present amendment, claims 1-47 will remain pending. The specification has been amended solely to correct typographical errors,

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remove hyperlink text, and to conform to U.S. format. No new matter has been added to the specification.

Applicants respectfully submit that the claims are in condition for allowance. An early notice of the same is earnestly solicited. The Examiner is invited to contact Applicants' undersigned representative at (215) 564-8906 if there are any questions regarding Applicants' claimed invention. Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

Respectfully submitted,



Paul K. Legaard

Registration No. 38,534

Date: **March 22, 2002**

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DOCKET NO.: BTGI-0025**PATENT****VERSION WITH MARKINGS TO SHOW CHANGES MADE****In the Specification:**

The following paragraph was inserted after the title on page 1:

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is the U.S. national phase of International Application No. PCT/EP00/09325 filed September 25, 2000, which claims priority to Application No. ES 9902474 filed November 11, 1999 and to Application No. ES 9902127 filed September 24, 1999, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The following header was inserted between lines 8 and 9 on page 1:

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Figure 3 shows an alignment of partial sequences from the TmDP sequence and a deduced amino acid sequence corresponding to an *Arabidopsis thaliana* genomic DNA sequence of previously unknown function.

The paragraph beginning at page 7, line 11 of the specification was amended as follows:

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Algorithms and software suitable for use in aligning amino acid or nucleotide sequences for comparison and calculation of sequence homology or identity will be known to those skilled in the art. Significant examples of such tools are the Pearson and Lipman search based FAST and BLAST programs. Details of these may be found in Altschul et al (1990), J. Mol. Biol. 215: 403-10; Lipman D J and Pearson W R (1985) Science 227, p1435-41. [Publically] Publicly available details of BLAST may be found on the internet at [<http://www.ncbi.nlm.nih.gov/BLAST/blast-help.html>] www.ncbi.nlm.nih.gov/BLAST/blast-help.html. Thus such homology and identity percentages can be ascertained using commercially or [publically] publicly available software packages incorporating, for example, FASTA and BLASTn software or by computer servers on the internet. Examples of the former are the GCG program package (Devereux et al [Nucleic] Nucleic Acids Research (1984) 12 (1): 387) and the Bestfit program (Wisconsin Sequence Analysis Package, eg. Version 8 for Unix or IBM equivalent, Genetics Computer Group, University [Research] Research Park, 575 Science Drive, Madison, WI 53711) which uses the local homology algorithm of Smith and Waterman, Advances in Mathematics, 2:482-489 (1981). Many international institutes, eg. Genbank (see [<http://www.ncbi.nlm.nih.gov/BLAST>] www.ncbi.nlm.nih.gov/BLAST) and EMBL: (see, [<http://www.embl-heidelberg.de/Blast2>] www.embl-heidelberg.de/Blast2), offer internet services.

In the Claims:

Claims 1-47 were amended as follows:

1. (Amended) A method of controlling one or more of plant growth, gene expression, cellular DNA replication, cell cycle progression, differentiation and development comprising increasing or decreasing E2F-dimerization partner (DP) protein activity in a plant cell through expression of a recombinant DP peptide or protein in [that] the plant cell, wherein [characterized in that] the peptide comprises [a sequence] SEQ ID No. 2, a functional part thereof, or a sequence having at least 70% homology to either, wherein the [that] peptide or protein [being] is capable of interacting with a plant E2F protein or peptide [such as] to alter E2F activity in the plant cell.

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2. (Amended) [A] The method [as claimed in] of Claim 1 [characterized in that] wherein the peptide or protein sequence is of 50% or more identity with that of the corresponding full length or part of SEQ ID No. 2.
3. (Amended) [A] The method [as claimed in] of Claim 1 [or Claim 2 characterized in that] wherein the peptide or protein sequence is of 70% or more identity with that of the corresponding full length or part of SEQ ID No. 2.
4. (Amended) [A] The method [as claimed in any one of the preceding claims characterized in that] of Claim 1 wherein the plant DP activity comprises one or both of (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate E2F binding to E2F/DP transcription factor binding sites in plant DNA.
5. (Amended) [A] The method [as claimed in any one of the preceding claims characterized in that it includes] of Claim 1 further comprising the steps of altering the plant DP protein level, the E2F-DP DNA-binding activity, transactivation properties, and/or DP/E2F-binding activity.
6. (Amended) [A] The method [as claimed in any one of the preceding claims characterized in that] of Claim 1 wherein the DP may be modified alone and/or in combination with a modification of the levels or activity of plant E2F and/or plant Rb.
7. (Amended) An isolated, enriched, cell free and/or recombinantly produced protein or peptide, capable of altering E2F-dimerization partner (DP) activity in a plant cell [, characterized in that it has] comprising one or both DP activities in plants selected from (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate [, particularly enhance,] E2F binding to E2F transcription factor binding sites in plant DNA or effect thereof, wherein [characterized in that] the protein or peptide comprises [an amino acid as shown in] SEQ ID No. 2 or a functionally active part thereof or a sequence having at least 70% homology to such sequence or part.

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8. (Amended) [A] The protein or peptide [as claimed in] of Claim 7 [characterized in that it has] comprising at least 50% identity to [the amino acid sequence as shown in] SEQ ID No 2 or a functional active part thereof.

9. (Amended) [A] The protein or peptide [as claimed in] of Claim 7 [or Claim 8 characterized in that it is of] comprising SEQ ID No 2 or [is] a [variants] variant thereof modified such that the amino acid sequence is mutated such that its ability to dimerize with E2F protein is reduced from that of the native sequence or abolished completely therefrom, whereby the peptide is capable of acting as a DP protein which decreases or abolishes native or recombinant E2F binding to its DNA binding site, thus inhibiting or abolishing E2F activity in a cell in which [is its] it is present.

10. (Amended) [A] The protein or peptide [as claimed in any one of the preceding Claims 7 to 9] of Claim 7 comprising [characterized in it comprises] a sequence found in [that of] SEQ ID No 2 or having at least 70% homology thereto selected from [those comprising] the group consisting of SEQ ID No 6 ARAAMAPPRGGAAAAATAALDLTGVIHLEAS SVPLPE RGGNAVQRKGAVDP, SEQ ID No 8 DKDRKKEKAAAPRITGWGLREYSKIVCEKVEAKGRTTYNEVADEIYSELKS, SEQ ID No 10 MAHIGQGFDEKNIRRRVYDAFNVLIALRVIAKEKKEIR WMGLSNRYEYKIK KLEEV, SEQ ID No 12 RKELVNKIRNKKALLQEIEKQFDDLQNIKLRNQTLLESS AENVNGIRL PFVLVKTSR, and SEQ ID No 14 KARVEIEISDDSKFAHFENGAPFTLHDDLSILEGVRGNS IG KAGRATLH.

11. (Amended) [A] The protein or peptide [as Claimed in] of Claim 10 [characterized in that] wherein the sequence comprises two or more of [these sequences] SEQ ID No 6, SEQ ID No 8, SEQ ID No 10, SEQ ID No 12, and SEQ ID No 14 or sequences at least 70% homologous thereto.

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12. (Amended) An [Isolated] isolated, enriched, cell free and/or recombinant nucleic acid comprising a sequence encoding for expression of a protein or peptide [as described in any one of Claims 7 to 11] of Claim 7.

13. (Amended) The nucleic [Nucleic] acid [as claimed in] of Claim 12 [characterized in that it includes] comprising the coding nucleic acid sequence of SEQ ID No 1 or a part thereof encoding for all or a functional part of the amino acid sequence shown therein [as defined above].

14. (Amended) The nucleic [Nucleic] acid [as claimed in] of Claim 12 [or Claim 13 as] wherein the nucleic acid is contained in plasmid pCLON33, deposit number CECT 5195 made on August 17[th], 1999[under the terms of the Budapest Treaty for the International Recognition of Microorganism Deposits for Patent Purposes of 28th April 1977 at the Coleccion Espanola de Cultivos Tipo].

15. (Amended) [A] The nucleic [acids as claimed in any one of Claims 12 to 14 characterized in that it] acid of Claim 12 wherein the nucleic acid encodes [for] a plant DP or a functional variant thereof [including the sequence of] and comprises SEQ ID No 1, [or] a sequence complementary [thereto] to SEQ ID No 1, or [otherwise] a sequence that is antisense [thereto] to SEQ ID No 1.

16. (Amended) A nucleic acid probe [characterized in that it comprises] comprising a DNA sequence [corresponding to] which encodes an amino acid sequence selected from the group consisting of SEQ ID No 2, SEQ ID No 4, SEQ ID No 6, and SEQ ID No [to] 8.

17. (Amended) A nucleic acid probe or primer [characterized in that it comprises] comprising a double or single stranded DNA [of] sequence [corresponding to] comprising 10 or more contiguous nucleotides [taken from the sequence] of SEQ ID No 1 with the proviso that the ten or more contiguous nucleotides are [it is] not selected from nucleotides encoding amino acids 70 to 136.

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[Such probes and primers may be used in Northern and Southern blotting and in PCR, including RT-PCR, and LCR.]

18. (Amended) An oligonucleotides probe [characterized in that it comprises] comprising at least 18 contiguous bases of [the] SEQ ID No. 1.

19. (Amended) [An] The oligonucleotide probe [as claimed in] of Claim 18 [characterized in that it is] comprising 30 to 100 bases [long].

20. (Amended) [An] The oligonucleotide primer [as claimed in] of Claim 17 [characterized in that it is of] comprising 10 to 20 bases [long].

21. (Amended) [Antisense] A DNA which is antisense to a nucleic [acids as claimed in any one of Claims 12 to 20] acid of claim 12.

22. (Amended) A nucleic acid [characterized in that it encodes] encoding the DP protein or peptide encoding sequence [as claimed in any one of Claims 7 to 11] of Claim 7 [together] with a sequence encoding an E2F protein or peptide.

23. (Amended) [A] The nucleic acid [as claimed in] of Claim 22 [characterized in that it] wherein the sequences encoding the DP and E2F [encoding sequences] are under control of the same regulatory element or elements.

24. (Amended) A nucleic acid vector or construct comprising a nucleic acid [as claimed in any one of Claims 12 to 23] of Claim 12 or antisense nucleic acid thereto.

25. (Amended) A plant cell comprising recombinant nucleic acid, a vector or a construct [as claimed in any one of Claims 12 to 24] of Claim 12.

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26. (Amended) A transgenic plant or part thereof comprising recombinant nucleic acid, a vector, DNA construct or cell [as claimed in any one of Claims 12 to 25] of Claim 12.

27. (Amended) An antibody [characterized in that it has been raised] against a DP peptide or protein [as claimed in any one of Claims 7 to 11] of Claim 7.

28. (Amended) [An] The antibody [as claimed in] of Claim 27 [characterized in that it] which is capable of specifically binding with plant DP factor peptides or proteins [as claimed in any one of Claims 7 to 11] of Claim 7, but not to the human, mouse or *Xenopus* DP.

29. (Amended) A method for identifying and/or isolating DNAs corresponding to complete or partial genes that are regulated in G1 passage, G1/S phase transition and/or S phase progression of the cell cycle [, said method] comprising contacting a sample of genomic DNA with a binding material specific for binding such complete or partial genes, removing non-bound DNA from the specific material [then], and releasing and isolating the bound DNA[.];

[characterized in that] wherein the specific binding material comprises a peptide or protein [including] comprising the DNA binding sequence of a protein that is capable of acting as a part of a plant hetero-oligomer transcription activator or repressor.

30. (Amended) [A] The method [as claimed in] of Claim 29 [characterized in that] wherein the specific binding material comprises a peptide or protein which [includes] comprises a plant E2F DNA binding domain.

31. (Amended) [A] The method [as claimed in] of Claim 30 [characterized in that] wherein the specific binding material comprises a peptide or protein which [includes] comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) hetero-dimerization domain and/or a plant retinoblastoma protein E2F binding domain.

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32. (Amended) [A] The method [as claimed in any one of Claims 29 to 31 characterized in that] of Claim 29 wherein the material comprises a peptide or protein that comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) binding domain together with a peptide or protein that [includes] comprises a plant dimerization partner (DP) E2F binding domain.

33. (Amended) [A] The method [as claimed in any one of Claims 29 to 32 characterized in that] of Claim 29 wherein the E2F and DP domains are comprised in Triticum monnocoocum sequences or functional variants or parts thereof.

34. (Amended) [A] The method [as claimed in] of Claim 33 [characterized in that] wherein the DP domains are comprised in peptides or proteins [as claimed in any one of Claims 7 to 11] of claim 7.

35. (Amended) [A] The method [as claimed in any one of Claims 29 to 34 characterized in that] of Claim 29 wherein the specific binding material peptide or protein is labeled or tagged to assist in identifying immobilizing it [, particularly] when in bound complex with the gene to be identified and/or isolated.

36. (Amended) [A] The method [as claimed in any one of Claims 29 to 35 characterized in that] of Claim 29 wherein the specific binding material comprises two peptides or proteins, one [including] comprising the E2F DNA binding domain, and one [including] comprising the DP hetero-dimerization domain bound together as a hetero-dimer.

37. (Amended) [A] The method [as claimed in any one of Claims 29 to 26 characterized in that] of Claim 29 wherein the E2F sequence is all or part of SEQ ID No 4 or a sequence having 90% homology thereto.

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38. (Amended) [A] The method [as claimed in any one of Claims 29 to 37 characterized in that] of Claim 29 wherein the binding material comprises a peptide or protein including an optionally labeled plant retinoblastoma protein E2F binding domain.

39. (Amended) A specific binding material [characterized in that it comprises] comprising a peptide or protein having DNA binding activity with respect to plant DNA transcription activator or repressor factor binding sites [, particularly in genomic DNA,] and having the ability to dimerize or oligomerize with a further such plant protein together with one or more of said further [a] peptides or proteins.

40. (Amended) A specific binding material [characterized in that it comprises] comprising
(i) a peptide or protein having DNA binding activity with respect to plant DNA E2F transcription factor binding sites [, particularly those present in genomic DNA] and having the ability to dimerize with plant DP protein together with one or both of

(ii) a peptide or protein that is capable of binding to plant E2F through its DP hetero-dimerization domain, and

(iii) a peptide or protein that is capable of binding to plant E2F through its retinoblastoma protein binding domain.

41. (Amended) [A] The material [as claimed in] of Claim 40 [characterized in that] wherein the peptides or proteins are wheat peptides or proteins, [and] functional variants thereof, or [and] parts thereof.

42. (Amended) [A] The material [as claimed in Claims 40 or 41 characterized in that it comprises] of Claim 40 comprising a hetero-dimer of (i) and (ii) or (i) and (iii).

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43. (Amended) [A] The material [as claimed in any one of Claims 40 to 42 characterized in that it comprises] of claim 40 comprising both of the peptides or proteins (i) and (ii) in the form of a hetero-dimer together with peptide or protein (iii).

44. (Amended) [A] The material [as claimed in any one of Claims 40 to 43 characterized in that] of Claim 40 wherein the peptides or proteins are in labeled or tagged form.

45. (Amended) [A] The material [as claimed in any one of Claims 40 to 44 characterized in that] of Claim 40 wherein it is in the form of a coating or otherwise bound form on a support material.

46. (Amended) [A] The material [as claimed in any one of Claims 40 to 44 characterized in that] of Claim 40 wherein the material is in the form of particles of the peptides or proteins and/or hetero-dimers or trimers.

47. (Amended) A nucleic acid [characterized in that it encodes for] encoding a peptide or protein [as claimed in any one of Claims 7 to 11] of Claim 7 fused to a sequence encoding [for] a protein label.

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WHEAT DP PROTEINS AND USES THEREOF

The present invention relates to novel nucleic acids and transcription factor proteins and peptides encoded thereby which have properties of modifying plant cell cycle when expressed or otherwise incorporated into plant cells. Such modification
5 can be used to manipulate plant and plant organ or tissue size or to overproduce specific gene products. Particularly provided are recombinant plant, and particularly wheat Triticum monococcum, DP protein transcription factor encoding nucleic acids, modified forms thereof and antisense nucleic acids thereto.

The present invention further provides methods for identifying and/or
10 isolating DNA responsive to said transcription factor proteins, particularly identifying and/or isolating such DNA in the form of promoters with or without open reading frames (ORFs) and other regulatory regions, eg. as whole or partial genes. It particularly relates to methods of identifying and/or isolating such ORFs, promoters and whole genes responsive to E2F in hetero-dimer form with its dimerization partner
15 protein (DP), and most particularly such DNA as found in plants. By identifying such DNAs in plants it is thus made possible using molecular biology and computational techniques to identify homologous DNAs of the same or similar function in other organisms, particularly animals, viruses and fungi, but also yeast and bacteria.

In animal cells, the retinoblastoma (Rb) protein is a major regulator of the cell
20 cycle transition from G1 to S phase. Rb exerts its function by regulating the activity of the so-called E2F family of transcription factors which control the expression of a set of genes required for G1 passage and the G1/S transition. In human cells, six E2F proteins have been identified (E2F-1 to-6). Full activity of E2F members depends of heterodimerization with other less-related members, the DP (dimerization partner)
25 proteins, of which two members (DP-1 and DP-2 or DP-3, depending on the nomenclature) have been identified.

In plant cells, the discovery, isolation and characterization of a maize Rb related protein (ZmRBR, formerly ZmRb1) revealed that a Rb-like pathway seems to be crucial for cell cycle transitions. Further studies strongly suggest that components

of this pathway might also be responsible for maintenance of particular differentiated states in plants.

WO 99/53075 (E.I. Du Pont de Nemours), incorporated herein by reference, discloses three Expressed Sequence Tags (ESTs) derived respectively from Impatiens
5 balsamina developing seed, from etiolated Corn seedlings 14 days after planting and from Soybean 8 day old root inoculated with eggs of cyst nematode Heterodera
glycines (Race 14) for 4 days (see Example 5 therein). These respective ESTs have relative low but apparently significant similarity to known Xenopus laevis (46%) and
10 Mus musculus (37%) and (48%) respectively. Percentage similarity between the three plant ESTs was found to be between 31% and 78%. That patent teaches that BLAST score and probabilities indicate that these sequences represent the first (identified) plant sequences encoding (part of) DP-1 proteins. That patent application further discloses ESTs encoding parts of putative plant DP-2 proteins and E2Fs.

WO 00/47614 (Pioneer Hi-Bred), incorporated herein by reference, was
15 published after the priority dates of the present application but before its filing date, and discloses a full cDNA and amino acid sequences for a Zea Mays DP protein, but does not designate this as of any of the sub-groups DP-1 to DP-3. The patent application provides a most comprehensive set of strategies and protocols for use of such cDNA and protein, but provides no description of them having actually been
20 expressed or produced. No properties for the putative DP protein are given other than sequence information and thus it would appear that the protein amino acid sequence at least is merely that deduced from the nucleotide sequence of the DNA.

The present applicant's copending patent application WO 99/58681 relates to recombinant DNA derived from Triticum monococcum that encodes for a protein
25 that acts as a plant E2F transcription factor. Although the determination of the nucleic acid sequence that will encode a functional plant E2F factor allows for transformation of plants such as to control cell cycle, and thus plant and specific plant organ and tissue size, it is thought to be possible that significantly high overexpression of E2F alone could be deleterious based on analogous animal

studies. These show that such expression might trigger an apoptotic-like pathway wherein plant cells would detect an increase in E2F and a concomitant entry in S-phase as abnormal.

5 In human systems binding of E2F to its binding sites is strongly stimulated (by over 50 fold) by presence of the DP protein together with human E2F in experimental systems. Binding of plant E2F to its responsive sites may be made more efficient and/or specific in the presence of such a plant DP. In human cells, a number of cell cycle and DNA replication-related genes have been shown to be expressed in an E2F-dependent manner. Thus the use of plant E2F as a trans-activator in plants would be
10 more efficient when combined with DP, particularly a plant DP, in the same plant if such a partner were to be available.

As yet a systematic, genome-wide search for all the E2F-responsive genes has not been carried out. One of the main reasons is that any protocol designed to randomly isolate DNA fragments containing E2F-binding sites does not identify the
15 promoter regions, where they are located and/or the genes that they regulate. Furthermore, the lack of whole genomic information is a drawback of such approaches. Attempts to identify potential E2F-binding sites have been carried out using the so-called CASTing (cyclic amplification and selection targets) system (Ouellette et al. (1992) Oncogene 7, 1075-1081; Tao et al., (1997) Mol. Cell Biol. 17,
20 6994-7007). This has allowed the identification of oligonucleotide sequences that contain E2F-binding sites but, again, no information on the promoters and where they are located can be extrapolated.

The Arabidopsis thaliana genome has now been largely made available, and is shortly to be fully sequenced, with for example an Arabidopsis sequence having
25 similarity to Homo-sapiens DP-2 protein genes having been deposited on 3rd April 2000 at EMBL under ID code ATT22P11. The availability of cDNA clones encoding functionally interacting plant E2F and DP proteins from the same plant species, ie. Triticum monococcum, provided now by the present inventors, offers a unique opportunity for a genome-wide search, identification and isolation of all E2F-binding

sites present in a plant genome. Such identified genes may then, for example, be used in BLAST searches of the Arabidopsis genome to establish existence of important, so far unidentified, universally applicable plant homologous gene families that may serve as targets for selection, molecular biological transformation and chemical agents.

The DP encoding DNA and protein encoded thereby provided by the present inventors are found to differ significantly in sequence from those of the prior art identified above. Thus, when compared using BLAST the present DP sequence has less than 45% identity and less than 60% similarity (homology) with the WO 99/53075 Impatiens DP1 EST where they overlap. When compared with the WO 99/53075 DP-2 EST from Zea Mays the identity found is less than 48% and the similarity is less than 58% where they overlap. When compared with the Triticum aestivum DP-2 EST identity found is less than 44% and similarity is less than 57%.

As described above, E2F and DP are two proteins that hetero-dimerize to form an active transcription factor that regulates the transition from G1 to S phase of the cell cycle and, later, the expression of genes required for S-phase progression. It is further known that E2F and retinoblastoma (Rb) proteins interact as a hetero-dimer in cells to repress certain genes. This repression involves binding of the retinoblastoma protein to the E2F-DP hetero-dimer that is in turn bound to sites on DNA through the E2F DNA binding domain. Thus certain important G1 repressed genes are thought to be identifiable best by a combination of E2F and Rb or Rb-E2F-DP together.

The determination of a functional plant DP amino acid sequence and the nucleic acid sequences encoding for this provides the possibility for transforming plants whereby E2F and DP levels or activity may be co-ordinated, thus avoiding any deleterious effects and allowing for increased options for plant growth regulation. Particularly this determination allows for the co-overexpression, co-underexpression or opposite sense expression of proteins or peptides having plant E2F and DP activity.

The uses of such recombinant nucleic acids are potentially numerous. For

example, some plant organs that need a period of proliferation to increase cell number before differentiation, e.g. such as buds for flowers, shoots and leaves, could be increased in size if expression of E2F and DP is co-ordinated and the proliferation period is extended for a few more cycles. Use of temporally
5 controllable promoters or naturally occurring modulators of expression of the recombinant nucleic acid would then allow the recombinant genes to be switched off and let differentiation occur. The descriptions of WO 99/53075 and WO 00/47614, incorporated herein by reference, provide further detailed instruction for uses for plant DP protein and encoding DNA that have equal application to the present
10 materials.

The present inventors have now isolated, cloned and characterized a nucleic acid comprising a wheat (Triticum monococcum-Tm) derived cDNA encoding a plant protein which interacts with plant E2F from wheat (Triticum monococcum-Tm) in the yeast two-hybrid system. They have established that this cDNA clone encodes a plant
15 E2F dimerization partner (DP) family member (TmDP) with amino acid regions having homology to conserved parts of animal DP proteins.

The present inventors have further provided a method for identifying and/or isolating DNAs corresponding to complete or partial genes that are regulated in G1 passage, G1/ S-phase transition and/or S phase progression of the cell cycle, said
20 method comprising contacting a sample of DNA, particularly whole genomic DNA that has been fragmented, eg by digestion or shearing, with a binding material specific for binding such complete or partial genes, removing non-bound DNA from the specific binding material then, releasing and isolating the bound DNA characterised in that the specific binding material comprises a peptide or protein including the DNA
25 binding sequence of a protein that is capable of acting as a part of a plant hetero-oligomer transcription activator or repressor, particularly as part of the wheat E2F/ DP heterodimer and most particularly is wheat Tm E2F/DP heterodimer.

With respect to the present specification and claims, the following technical terms are used in accordance with the definitions below.

A "functional variant" of a peptide or protein is a polypeptide the amino acid sequence of which can be derived from the amino acid sequence of the original peptide or protein by the substitution, deletion and/or addition of one or more amino acid residue in a way that, in spite of the change in the amino acid sequence, the functional variant retains at least a part of at least one of the biological activities of the original protein that is detectable for a person skilled in the art. A functional variant is generally at least 50% homologous, advantageously at least 70% homologous and even more advantageously at least 90% homologous to the protein from which it can be derived. Preferably the amino acid sequence of the functional variant is 50% identical, more preferably 70% identical and most preferably 90% identical to the peptide or protein. Any functional part of a protein or a variant thereof is also termed functional variant. Further preferred conservatively substituted variants are as defined in WO 00/47614, pages 7-8. Biological function in the present application is the ability to bind to and or alter the DNA binding capabilities of E2F proteins.

By homologous is meant that the stated percentage of the amino acid sequence has identity or is of conservatively substituted amino acids. By identical is meant that the stated percentage of the amino acid sequence is identical. Both these percentage terms allow for gapping of sequences to allow alignment as is described below.

Particularly, by the term identity is meant that the stated percentage of the claimed amino acid sequence or base sequence is to be found in the reference sequence in the same relative positions when the sequences are optimally aligned, notwithstanding the fact that the sequences may have deletions or additions in certain positions requiring introduction of gaps to allow alignment of the highest percentage of amino acids or bases. Preferably the sequence are aligned by using 20 or less gaps, ie. the total number of gaps introduced into the two sequences when added together is 20 or less, more preferably 10 or less. The length of such gaps is not of particular importance as long as one or other of the two defined activities relating to E2F binding and E2F DNA binding modulation is retained but generally will be no more than 50, and preferably no more than 10 amino acids, or 150 and preferably no more than 30 bases.

5 Variants from the aforesaid sequences preferably are conservative substitutions. The expression 'conservative substitutions' as used with respect to amino acids relates to the substitution of a given amino acid by an amino acid having physicochemical characteristics in the same class. Thus where an amino acid has a hydrophobic characterising group, a conservative substitution replaces it by another amino acid also having a hydrophobic characterising group; other such classes are those where the characterising group is hydrophilic, cationic, anionic or contains a thiol or thioether. Such substitutions are only contemplated where the resultant protein has activity as a DP peptide or protein as discussed with respect to E2F heterodimerization and modulation of E2F-DNA binding or transcription activation.

10 Algorithms and software suitable for use in aligning amino acid or nucleotide sequences for comparison and calculation of sequence homology or identity will be known to those skilled in the art. Significant examples of such tools are the Pearson and Lipman search based FAST and BLAST programs. Details of these may be found in Altschul et al (1990), J. Mol. Biol. 215: 403-10; Lipman D J and Pearson W R (1985) Science 227, p1435-41. Publically available details of BLAST may be found on the internet at 'http://www.ncbi.nlm.nih.gov/BLAST/blast-help.html'. Thus such homology and identity percentages can be ascertained using commercially or publically available software packages incorporating, for example, FASTA and BLASTn software or by computer servers on the internet. Examples of the former are the GCG program package (Devereux et al Nucleic Acids Research (1984) 12 (1): 387) and the Bestfit program (Wisconsin Sequence Analysis Package, eg. Version 8 for Unix or IBM equivalent, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) which uses the local homology algorithm of Smith and Waterman, Advances in Mathematics 2:482-489 (1981). Many international institutes, eg. Genbank (see http://www.ncbi.nlm.nih.gov/BLAST) and EMBL: (see http://www.embl-heidelberg.de/Blast2), offer internet services.

25 Parameters used in with software packages and internet servers should be applied with the appropriate sequence lengths and aforesaid gap characteristics in

mind. Alignment strategies are discussed further in WO 98/40483 on pages 39 to 41, which document is incorporated herein by reference

Convenient parameters for BLAST searches are the default values, ie. for EMBL Advanced Blast2: Blastp Matrix BLOSUMS 62, Filter default, Echofilter X, Expect 10, Cutoff default, Strand both, Descriptions 50, Alignments 50. For BLASTn defaults are again preferably used. GCG Wisconsin Package defaults are Gap Weight 12, Length weight 4. FASTDB parameters used for a further preferred method of homology calculation are mismatch penalty = 1.00, gap penalty =1.00, gap size penalty = 0.33 and joining penalty =30.0.

10 The term "overproducing" is used herein in the most general sense possible. A special type of molecule, usually a polypeptide or an RNA, is said to be "overproduced" in a cell if it is produced at a level significantly and detectably higher (e.g. 20% higher) than natural level, ie. that found in a cell of the same lineage that has not been transformed with plant the DNA described herein. Overproduction of a molecule in a cell can be achieved via both traditional mutation and selection techniques and genetic manipulation methods as long as one of the parents used in selection methods is of transgenic type. The term 'underproducing' is intended to cover production of polypeptide or mRNA at a level significantly lower than the natural level (eg. 20% or more lower), but particularly to undetectable levels.

20 The term "ectopic expression" is used herein to designate a special realisation of overproduction in the sense that, for example, an ectopically expressed peptide or protein is produced at a spatial point of a plant where it is naturally not at all (or not detectably) expressed, that is, said peptide or protein is overproduced at said point. Particularly preferred ectopic expression is that which only reaches functional levels in a selected tissue and does not do so throughout the plant. This preferred ectopic expression is in contrast to constitutive expression.

25 A "gene" is a DNA sequence that controls a discrete hereditary characteristic and as such is not limited to DNA coding for proteins but relates to ORFs together with any regulatory sequence thereof. Thus a partial gene or part of a gene may include no ORF sequence, but consist only of regulatory, eg. a promoter, or associated

sequence, eg introns, particularly being in the present case that sequence which is subject to transcription factor specific binding, particularly by E2F-DP hetero-dimer and Rb-E2F-DP tertiary complex.

The expression 'conservatively substituted' as used with respect to amino acids
5 relates to the substitution of a given amino acid by an amino acid having physicochemical characteristics in the same class. Thus where an amino acid has a hydrophobic characterising group, a conservative substitution replaces it by another amino acid also having a hydrophobic characterising group; other such classes are those where the characterising group is hydrophilic, cationic, anionic or contains a
10 thiol or thioether. Such substitutions are only contemplated where the resultant protein has activity as a DP peptide or protein as discussed with respect to DNA and E2F dimerization.

Nucleic acids of the invention may be degeneratively substituted with respect to that exemplified herein in the sequence listing. The expression 'degeneratively
15 substituted' refers to substitutions of nucleotides by those which result in codons encoding for the same amino acid; such degenerative substitutions being advantageous where the cell or vector expressing the protein is of such different type to the DNA source organism cell that it has different codon preferences for transcription/translation to that of the cDNA source cell. Such degenerative
20 substitutions will thus be host specific.

Disclosure of the Invention

In a first aspect of the present invention there is provided a method of controlling one or more of plant growth, gene expression, cellular DNA replication,
25 cell cycle progression, differentiation and development comprising increasing or decreasing E2F-dimerization partner (DP) protein activity in a plant cell through expression of a recombinant DP peptide or protein in that cell characterised in that the peptide or protein comprises a sequence SEQ ID No 2, a functional part thereof, or a sequence having at least 70% homology to either, that peptide or protein being

capable of interacting with a plant E2F protein or peptide such as to alter E2F activity in the plant cell.

Preferably the peptide or protein is of 50% or more identity with that of the corresponding full length or part of SEQ ID No 2, more preferably 70% identity and
5 most preferably 90% identity. More preferably the peptide or protein is of 90% homology with the full length or part of SEQ ID No 2.

Preferably the method is characterised in that the plant DP activity comprises one or both of (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate, particularly enhance, E2F binding to E2F/DP transcription factor binding
10 sites in plant DNA.

The method may include steps of altering the plant DP protein level, the E2F-DP DNA-binding activity, transactivation properties, and/or the DP/E2F-binding activity. The plant DP may be modified alone and/or in combination with a modification of the levels or activity of plant E2F and/or plant Rb. The ability to
15 enhance E2F binding to the E2F transcription factor binding sites in plant DNA need not necessarily lead to transcription activation. Binding of the E2F-DP heterodimer with inhibition of such activation can also be provided using the present invention as can be decreased E2F-DNA binding or transcription.

Particularly the method may be used to alter plant cell, organ or tissue shape, and it may particularly alter cell proliferation characteristics such as to increase or
20 decrease plant cell, organ or tissue size. The method may also increase or decrease expression of other proteins with transformed cells and cells derived therefrom, particularly direct or indirect progeny.

In a second aspect the present invention provides an isolated, enriched, cell
25 free and/or recombinantly produced protein or peptide, capable of altering E2F-dimerization partner (DP) activity in a plant cell, characterised in that it has one or both DP activities in plants selected from (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate, particularly enhance, E2F binding to E2F transcription factor binding sites in plant DNA or effect thereof.

characterised in that the protein or peptide comprises an amino acid as shown in SEQ ID No 2 or a functionally active part thereof or a sequence having at least 70% homology to such sequence or part, more preferably at least 90% homology thereto and most preferably having at least 50% identity therewith, or still more preferably having at least 70% or at least 90% identity therewith.

More preferably the peptide or protein comprises at least 50% of the contiguous sequence and still more preferably at least 70% thereof.

One group of possible peptides or proteins of the invention are characterised in that they are of SEQ ID No 2 or variants thereof modified such that the amino acid sequence is mutated such that its ability to dimerize with E2F protein is reduced from that of the native sequence or abolished completely therefrom, whereby the peptide is capable of acting as a DP protein which decreases or abolishes native or recombinant E2F binding to its DNA binding site, thus inhibiting or abolishing E2F activity in a cell in which is its present.

Preferred peptides or proteins of the invention are further characterised in that they comprises a sequence found in that of SEQ ID No 2 or having at least 70% homology thereto selected from those comprising

SEQ ID No 6 ARAAMAPPRGGAAAAATAALDLTGVMHILEAS SVPPLPE
RGGNAVQRKGAVDP

SEQ ID No 8 DKDRKKEKAAAPRITGWGLREYSKIVCEKVEAKGRT TY
NEVADEIYSELKS

SEQ ID No 10 MAHIGQGFDEKNIRRRVYDAFNVLIALRVIAKEKKEIR
W MGLSNRYEYKIKKLEEV

SEQ ID No 12 RKELVKNKIRNKKALLQEIEKQFDDLQNIKLNRNQTLESS A
ENVNGIRLPFVLVKTSR

SEQ ID No 14 KARVEIEISDDSKFAHFENGAPFTLHDDL SILEGVRGNS
IGKAGRATLH

Most preferably the sequence comprises two or more of these sequences or sequences at least 70% homologous, more preferably at least 90% and still more

preferably at least 95% homologous thereto, still more preferably being the stated at least percentages identical.

Particularly useful peptides or proteins comprise at least one or more of the sequences of SEQ ID No 2 or percentage homologous or percentage identical
5 sequences thereto comprising amino acids 70 to 136 (the so called DNA binding domain), amino acids 137 to 200, (the heterodimerization domain) and amino acids 55 to 62 (the putative nuclear localization signal), more preferably two or more of these.

For some purposes it will be convenient to provide peptides or proteins of reduced length, for example 16 to 300, more preferably from 16 to 100 amino acids.

10 Useful variants of such proteins however are those in which non-essential or essential amino acids for E2F dimerization are modified, eg. by site directed mutagenesis, eg using PCR.

Particularly useful is nucleic acid the expression of which is controlled using tissue specific or chemically inducible promoters

15 A third aspect of the present invention provides isolated, enriched, cell free and/or recombinant nucleic acid comprising a sequence encoding for expression of a protein or peptide as described in the first aspect of the invention. Preferred nucleic acids comprise DNA of less than 4,000 basepairs. Preferred nucleic acids comprise only one peptide or protein encoding DNA sequence, optionally together with a
20 reporter gene.

Preferably the nucleic acid is that encoding for a plant DP or a functional variant thereof including the coding nucleic acid sequence of SEQ ID No 1 or a part thereof encoding for all or a functional part of the amino acid sequence shown therein as defined above. Preferred nucleic acid comprises DNA or RNA wherein when the
25 nucleic acid is RNA the base T is substituted by U.

A nucleic acid encoding for a TmDP of sequence of SEQ ID No 1 has been deposited on August 17th 1999 under the terms of the Budapest Treaty for the International Recognition of Microorganism Deposits for Patent Purposes of 28th April 1977 at the Coleccion Espanola de Cultivos Tipo in plasmid pCLON33 under

deposit number CECT 5195. Restriction enzymes contained in the multicloning site of the plasmid can be used to excise the insert cDNA from this, eg. EcoRI and XhoI or BamHI and XhoI. For in vitro transcription-translation, the full-length TmDP DNA was cloned into pBluescriptSK+ using BamHI and XhoI restriction enzymes.

5 It will be understood that nucleic acids of the invention may be double stranded DNAs or single stranded DNA of the cDNA or a sequence complementary thereto, eg. such as will have use as a probe or primer.

 Preferred nucleic acids are characterised in that they encode for a plant DP or a functional variant thereof including the sequence of SEQ ID No 1 or a sequence
10 complementary thereto. Further preferred nucleic acids comprise DNA, whether double or single stranded, sense, complementary or otherwise antisense thereto. Preferred nucleic acids comprise a cDNA optionally provided together with promoter, enhancer or stop sequences with no other gene coding regions. .

 The DNA or RNA of the invention may have a sequence containing
15 degenerate substitutions in the nucleotides of the codons in the sequences encoding for DP proteins or peptides of the invention. In RNA U's replace the T's of DNA. Preferred *per se* DNAs or RNAs are capable of hybridising with the polynucleotides encoding for peptides or proteins of the invention in conditions of low stringency, being preferably also capable of such hybridisation in conditions of high stringency.

20 The terms "conditions of low stringency" and "conditions of high stringency" are of course understood fully by those skilled in the art, but are conveniently exemplified in US 5202257, columns 9 and 10 and in WO 98/40483 on page 3; both of which are incorporated herein by reference. Thus, generally, the most preferred nucleic acids of the invention will hybridise at the most stringent conditions described
25 in these patents while other embodiments will hybridise at the milder stringency or low stringency conditions. Further examples of preferred stringency are described in PCT/IB97/00409, see page 21, line 23 to page 27, line 15 and the corresponding US patent application, incorporated herein by reference.

 In US 5202257 low-stringency conditions comprise a temperature of about

37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (SSC) concentration; or, alternatively, a temperature of about 50°C or less, and a moderate to high salt (SSPE) concentration. The preferred conditions for such screening comprise a temperature of about 37°C, a formamide concentration of about 5 20%, and a salt concentration of about 5 times standard saline citrate (SSC; 20 times. SSC contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0); or a temperature of about 50°C, and a salt concentration of about 2 times SSPE (1 times SSPE contains 180 mM NaCl, 9 mM Na₂ HPO₄, 0.9 mM NaH₂PO₄ and 1 mM EDTA, pH 7.4.

High stringency conditions are described as comprising a temperature of about 10 42°C or less, a formamide concentration of less than about 20%, and a low salt (SSC) concentration; or, alternatively, a temperature of about 65°C or less, and a low salt (SSPE) concentration. The preferred conditions for such screening are described as comprising a temperature of about 42°C, a formamide concentration of about 20%, and a salt concentration of about 2.times. SSC; or a temperature of about 65°C, and a 15 salt concentration of about 0.2 times SSPE.

WO 00/47614 also describes conditions of stringent hybridization conditions of high, moderate and low nature and these are found on its page 17-18, incorporated herein by reference. These are thus further conventional equivalents for use in hybridaizations and are incorporated herein by reference for the purpose pf providing 20 alternative option for identifying suiatble sequences.

Where modifications are made they should lead to the expression of a protein with different amino acids in the same class as the corresponding amino acids to these DP peptide or protein sequences; that is to say, they are conservative substitutions. Such substitutions are known to those skilled in the art see, for example, US 5380712 25 which is incorporated herein by reference, and are considered only when the protein is active as a DP peptide or protein with regard to its interactions with E2F as an E2F-DP heterodimer.

DNA or RNA provided from a plant or the deposit referred to above may be altered by mutagenic means such as the use of mutagenic polymerase chain reaction

primers. Methods of producing the proteins or peptides of the invention characterised in that they comprise use of the DNA or RNA of the invention to express them from cells are also provided in this aspect. Examples of probes are the DNA sequences corresponding to amino acid sequences SEQ ID No 2 to 8 above.

5 For the purpose of screening for plant DPs, a process which has heretofore been hampered due to human E2F and DP dissimilarity to plant E2F and DP, nucleic acid probes or primers comprising a double or single stranded DNA of sequence corresponding to 10 or more contiguous nucleotides taken from the sequence SEQ ID
10 No 1 are provided, with the proviso that they are not selected from those just encoding for the amino acid sequence that is relatively highly conserved with human DP, ie the DNA binding region of amino acid 70 to 136 is the most conserved region. Such probes and primers may be used in Northern and Southern blotting and in PCR, including RT-PCR, and LCR.

Oligonucleotides for use as probes conveniently comprise at least 18
15 contiguous bases of the sequences of the invention, preferably being of 30 to 100 bases long, but may be of any length up to the complete sequence or even longer. For use as PCR or LCR primers the oligonucleotide preferably is of 10 to 20 bases long but may be longer. Primers should be single stranded but probes may be also be double stranded ie. including complementary sequences.

20 For the purpose of downregulating native plant DP expression there is also provided antisense DNA to any of the nucleic acids of the invention described above. This technique is well known in the art but is generally illustrated by US 5356799 and US 5107065 by way of example, each of which is incorporated herein by reference. Anti-sense DNA is of length sufficiently long enough such that when expressed as
25 RNA that downregulates expression of native or recombinant DP to levels that are measurably lower, eg. such that DP mRNA in Northern blots is lower or not measurably present.

A preferred form of the nucleic acid of the invention provides the DP protein or peptide encoding sequence as described above together with a sequence encoding

the E2F protein or peptide. Such sequences are conveniently and sometimes advantageously under control of the same regulatory element or elements, eg. promoters, such that they may act together as a heterodimer.

5 A fourth aspect of the invention provides a nucleic acid vector or construct comprising a nucleic acid of the present invention or comprising antisense nucleic acid thereto. Suitable vectors or constructs for introducing the peptides or proteins of the invention into plants will occur to those skilled in the art of plant molecular biology, but are conveniently those discussed below with respect to methods for producing transgenic plants. Such vector or construct may thus also comprise both DP
10 and E2F peptides or proteins or antisense or other related sequences as described above.

A fifth aspect of the present invention provides a plant cell comprising recombinant nucleic acid, preferably recombinant DNA, of the third aspect of the invention. Nucleic acids of the invention are particularly provided in the form of such
15 nucleic acid vectors or DNA construct comprising that nucleic acid or antisense nucleic acid sequence thereto.

A sixth aspect of the present invention provides a plant cell comprising antisense nucleic acid thereto capable of downregulating expression of native plant DP.

20 A seventh aspect of the present invention comprises a transgenic plant or part thereof comprising recombinant nucleic acid, a vector, DNA construct or cell as described above.

It will be realised that a most effective method of delivering proteins and peptides of the invention to plant cells is by expressing nucleic acid encoding them *in situ*. Such method is conventionally carried out by incorporating oligonucleotides or
25 polynucleotides, having sequences encoding the peptide or protein, into the plant cell DNA. Such nucleotides can also be used to downregulate native DP expression by gene silencing coexpression or through antisense strategy. By use of mutagenesis techniques, eg. such as SDM, the nucleotides of the invention may be designed and

produced to encode proteins and peptides which are functional variants or otherwise overactivated or inactivated, eg. with respect to binding, of the invention

Preferred plants of the seventh aspect may comprise the nucleic acid of the invention in a construct in functional association with promoter, activating or
5 otherwise regulating sequences. Preferred promoters may be tissue specific such that the resultant expression of peptide, and thus its effects, are localised to a desired tissue. Promoters with a degree of tissue specificity will be known to those skilled in the art of plant molecular biology. Some of these are discussed below.

Methods of producing isolated or purified DNA/RNA, vectors and constructs
10 capable of being used in the present invention will occur to those skilled in the art in the light of conventional molecular biology techniques. DNA, RNA and vector containing or encoding for these may be introduced into target cells in known fashion in the field of plant cell transformation. For example the method of introducing the DNA or RNA into cells, which eg. may be somatic or pollen cells, using techniques
15 such as electroporation or gene gun technology.

It may be preferred to express the DNA or RNA of the invention throughout the plant, but in the event that tissue specific effect is to be exploited then it will be understood by those skilled in the art that tissue specific promoters, enhancers or other activators should be incorporated into the transgenic cells employed in operative
20 relation with the DNA.

It will be realised by those skilled in the art that suitable promoters may be active ectopically, continuously or may be inducible. It will be appreciated by those skilled in the art that inducible or tissue specific ie promoters will have advantage in so far as they are capable of providing alteration of the aforesaid DP peptide or
25 protein activity only when or where required, eg. at a particular stage of cell development or in a tissue such as leaves, roots, fruit or seeds or subparts thereof, eg. endosperm, that may be the subject of desired increase or decrease in size or even deletion.

No particular limitation on the type of promoter to be employed is envisioned,

although a reasonable amount of experimental trial may be expected to be undertaken to produce good results. Examples of tissue specific and inducible promoters can be found in the following patent literature: US 5086169 (pollen specific), US 5459252 and US 5633363 (root specific), US 5097025 ((i)seed, (ii)mature plant), US 5589610
5 (stamen), US 5428146 (wound), US 5391725 ((i)chloroplast, (ii) cytosol), US 4886753 (root nodule), US 4710461 (pollen), US 5670349 (pathogen), US 5646333 (epidermis), US 5110732 ((i) root , (ii) radical), US 5859328 (pistil), US 5187267 (heat shock), US 5618988 (storage organ), US 5401836 and US 5792925 (root), US 4943674 (fruit), US 5689044 and US 5654414 (chemical), US 5495007 (phloem), US
10 5589583 (meristem), US 5824857 (vasculature), each of which is incorporated herein by reference. Constitutive promoters will be well known to those skilled in the art and are discussed in the documents above and referred to below but for example include CaMv35S and alfalfa (MsH3g1) (see WO 97/20058 incorporated herein by reference).

15 Numerous specific examples of methods used to produce transgenic plants by the insertion of cDNA in conjunction with suitable regulatory sequences will be known to those skilled in the art. Plant transformation vectors have been described by Denecke et al (1992) EMBO J. 11, 2345-2355 and their further use to produce transgenic plants producing trehalose described in US Patent Application Serial No.
20 08/290,301. EP 0339009 B1 and US 5250515 describe strategies for inserting heterologous genes into plants (see columns 8 to 26 of US 5250515). Electroporation of pollen to produce both transgenic monocotyledonous and dicotyledonous plants is described in US 5629183, US 7530485 and US 7350356. Further details may be found in reference works such as Recombinant Gene Expression Protocols. (1997)
25 Edit Rocky S. Tuan. Humana Press. ISBN 0-89603-333-3; 0-89603-480-1. All of these documents are incorporated herein by reference It will be realised that no particular limitation on the type of transgenic plant to be provided is envisaged; all classes of plant, monocot or dicot, may be produced in transgenic form incorporating the nucleic acid of the invention such that DP activity in the plant is altered,
30 constitutively or ectopically.

In an eighth aspect of the present invention the present inventors make available antibodies capable of specifically binding with plant DP factor peptides or proteins of the first aspect of the present invention, thus enabling the identification and isolation of further peptides and proteins of the invention and nucleic acid sequences encoding therefor, eg. using techniques such as Western blotting. Preferably these antibodies are selected such that they do not bind to the other DPs described in Figure 2, eg. by selection for absence of significant binding to a column on which these are mounted as ligand binding agents. Such antibodies are provided by use of oligopeptides and polypeptides consisting of parts of the TmDP that are not replicated in mammalian DPs, ie. not the so called DNA binding domain specified above, to raise the antibody eg in rats or rabbits etc..

In a ninth aspect of the invention there is provided the aforesaid method for identifying and/or isolating DNAs corresponding to complete or partial genes that are regulated in G1 passage, G1/S-phase transition and/or S phase progression of the cell cycle, said method comprising contacting a sample of DNA, particularly whole genomic DNA that has been fragmented, eg by digestion or shearing, with a binding material specific for binding such complete or partial genes, removing non-bound DNA from the specific binding material then, releasing and isolating the bound DNA.

characterised in that the specific binding material comprises a peptide or protein including the DNA binding sequence of a protein that is capable of acting as a part of a plant hetero-oligomer transcription activator or repressor.

Preferably the method is characterised in that the specific binding material comprises a peptide or protein which includes a plant E2F DNA binding domain, however, other plant hetero-oligomer transcription activator protein DNA binding sequences may be used, eg, the GRAB proteins of PCT/EP98/03662 incorporated herein by reference.

The specific binding material particularly preferably comprises a peptide or protein which includes a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) hetero-dimerization domain and/or a plant retinoblastoma protein E2F binding domain.

Most advantageously the material comprises a peptide or protein that comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) binding domain, hereinafter called the E2F hetero-dimerization domain, optionally together with a peptide or protein that includes a plant dimerization partner (DP) E2F binding domain, hereinafter called the DP hetero-dimerization domain.

Preferably these are both the TmDP and TmE2F or functional variants or parts thereof as defined above having the set defined homology and/or identity.

Preferably the specific binding material peptide or protein is labelled or tagged to assist in identifying or immobilising it, particularly when in bound complex with the gene to be identified and/or isolated. More preferably, and particularly advantageously, the specific binding material comprises two peptides or proteins, one including the E2F DNA binding domain, and one including the DP hetero-dimerization domain bound together as a hetero-dimer.

The peptide or protein including the E2F DNA binding domain may consist of a complete plant E2F protein, optionally but preferably labelled, but may consist of only a part thereof, eg. just the DNA binding domain, the DNA binding domain and the hetero-dimerization domain or larger peptides or proteins including these, eg. truncates of plant E2F. Suitable peptides and proteins are described in copending PCT/EP99/03158 (incorporated herein by reference) and include all the functional variants including the E2F binding domain disclosed therein. Preferably the peptide or protein is a truncated or whole wheat E2F protein as disclosed in that patent and having all or part of SEQ ID No 4 shown below, preferably conjugated with a label.

The peptide or protein including the DP hetero-dimerization domain may consist of a complete plant DP protein, optionally but preferably labelled, but may consist of only a part thereof, eg. just the hetero-dimerization-domain or larger peptides and proteins including this, eg. truncates of plant DP. Suitable peptides and proteins include functional variants. Preferably the peptide or protein is a truncated or whole wheat DP protein, having all or part of the amino acid sequence SEQ ID No 2 shown below, or a functional variant thereof, preferably conjugated with a label.

The binding material may alternatively or additionally incorporate a peptide or protein including a plant retinoblastoma protein E2F binding domain, hereinafter called a plant retinoblastoma (Rb) hetero-dimerization domain. Again, this may take the form of a whole plant retinoblastoma protein, optionally but preferably labelled,
5 but may be a truncate thereof, or a functional variant of one of these.

For the purpose of merely identifying genes and parts thereof which bind with E2F on its own, a peptide or protein amino acid sequence comprising only the DNA binding domain of E2F may be present, optionally in a peptide or protein including other non-functional sequence. However, most advantageously two peptides or
10 proteins will be present and will include the respective E2F and DP hetero-dimerization domains with the E2F DNA binding domain in order that the full enhancing effect of DP on E2F binding may be provided. It may be foreseen that use of a peptide or protein including the E2F DNA binding and hetero-dimerization domains together with a peptide or protein including the DP hetero-dimerization
15 domain without the DNA binding enhancing domain may be of interest for some genes, it may be preferred to include also the DP DNA binding enhancing domain.

Labelling of the respective peptide or proteins may in principle be made with conventional labelling material, but advantageously these should be different for each of the peptides or proteins used. Conveniently the label is in the form of a fusion
20 partner, eg, the peptide or proteins are provided as GST or MBP fusion peptide or proteins. Other forms of labelling will occur to those skilled in the art in the light of these. Use of fusions such as GST and MBP 'tags' allows for immobilisation of the peptide or protein on Gltathione Sepharose and on Amylose resins respectively.

Using the DNAs isolated by the present method it is possible to obtain their
25 sequences and, by using computational homology analysis of other organism's genome eg of plant, animal, yeast, bacteria, virus or fungal genomes, or molecular biology probing or other analysis techniques to identify therapeutic or otherwise industrially useful target genes involved in cell cycle that are no readily obtainable due to the comparative complexity or lack of information of/on such systems.

The present inventors have cloned, over-expressed in E. coli and purified both E2F (wheat E2F) and DP (wheat, E2F) as GST and MBP fusion proteins, respectively. They have shown these to hetero-dimerize *in vitro* and the corresponding tags do not seem to interfere with this interaction. Furthermore, binding of E2F to a double-stranded oligonucleotide containing the consensus sequence for human E2F-1 is stimulated ~20-50-fold by its association with DP in hetero-dimer form.

A preferred method of the invention comprises a procedure for isolating DNA fragments containing E2F-binding sites wherein a column of the specific binding material, eg. the purified labelled E2F-DP hetero-dimer, is prepared and a solution holding a DNA sample to be screened for genes and gene parts is passed down it. Conveniently a GST-E2F/MBP-DP hetero-dimer is prepared, is used to coat a support material such as glass beads in order to maximise surface area, and used to load a column. The DNA sample is advantageously a digested purified genomic DNA from a plant, eg. Wheat or Arabidopsis, the digestion having been carried out with a frequent cutter providing convenient DNA ends, e.g. a Sau3A cutter. Alternatively, genomic DNA can be sheared to a size ranging from 200 to 500 base pairs.

In a further preferred method the DNA sample is bound batchwise to the labelled hetero-dimer binding material, eg. on a support material or in free form, suspended in a buffer used routinely for binding experiments.

Washing of the column or batch of binding material is carried out extensively to remove non-specific binding DNAs before the specifically bound DNA is eluted with a high salt solution. If this removal is not complete, elution of the protein-DNA complexes may be carried out and the DNA may be purified in a separate step.

A library may be made of all bound DNA fragments and the inserts sequenced. Using conventional bioinformatic tools, the chromosomal location of each DNA fragment may be determined. The corresponding analysis of E2F/DP-dependent promoter activity may be determined using convenient reporter genes.

This procedure provides the capability to produce a virtually complete list of all the E2F, E2F/DP, Rb/E2F and/or Rb/E2F/DP responsive or repressed genes in the

genome of a multi-cellular organism, a plant in particular, something that as yet has been unachievable. In addition to the genes known to respond or bind to E2F/DP or Rb/E2F or Rb/E2F/DP in human cells, the present method can identify new or known genes in plants whose promoters are not suspected to be dependent on E2F and this
5 information can also be used to identify counterparts in human cells.

Particularly the present method is characterised in that the plant DP activity comprises one or more of (i) the ability to dimerize with plant E2F protein and (ii) the ability to enhance or decrease E2F binding to E2F/DP transcription factor binding sites in plant DNA. The ability to enhance E2F binding to the E2F transcription factor
10 binding sites in plant DNA need not necessarily lead to transcription activation.

Particularly the genes, promoters and ORFs provided by the present method may be used in recombinant form to alter cell, organ or tissue shape, particularly in plants but also in other organisms, and it may particularly alter cell proliferation characteristics such as to increase or decrease plant cell, organ or tissue size. The
15 method may also increase or decrease expression of other proteins with transformed cells and cells derived therefrom, particularly direct or indirect progeny.

In a tenth aspect the present invention provides a specific binding material characterised in that it comprises

(i) a peptide or protein having DNA binding activity with respect to plant
20 DNA transcription activator or repressor factor binding sites, particularly in genomic DNA, and having the ability to dimerize or oligomerize with a further such plant protein together with one or more of said further a peptides or proteins.

Preferably the specific binding material is characterised in that it comprises

(i) a peptide or protein having DNA binding activity with respect to plant
25 DNA E2F transcription factor binding sites, particularly those present in genomic DNA and having the ability to dimerize with plant DP protein together with one or both of

(ii) a peptide or protein that is capable of binding to plant E2F through its DP hetero-dimerization domain and

(iii) a peptide or protein that is capable of binding to plant E2F through its retinoblastoma protein binding domain.

Preferably these are the wheat peptide or proteins and functional variants and parts thereof as define above.

5 Preferably the material comprises a hetero-dimer of (i) and (ii) or (i) and (iii). Preferably the binding material comprises both of the peptides or proteins (i) and (ii), most preferably as a hetero-dimer. The material may also comprise the peptides or proteins (i) and (ii) in the form of a hetero-dimer together with peptide or protein (iii). Most preferably peptides or proteins (i), (ii) and/or (iii) are in labelled or tagged form,
10 particularly preferably being labelled through being fused with a tag eg. a fusion peptide or protein, advantageously independently, for example with GST or MBP.

 In a preferred form of this aspect the binding material is provided in the form of a coating or otherwise bound form on a support material, eg. on glass beads, phosphocellulose, sepharose or amylose or some similar support such as is used on an
15 affinity column, or particularly being in the form of an elutable column filled with the peptide or protein (i) and (ii) and/or (iii), particularly as said independently double labelled hetero-dimers comprising said fusion peptides or proteins and/or as a coating on support material. Alternatively the material is in the form of particles, eg. granules or spheroids, of the peptides or proteins and/or hetero-dimers described above.

20 As stated previously the DP, E2F and Rb peptides or proteins may be truncates and/or variants of the respective parts of the plant DP and E2F proteins set out in SEQ ID No 2 and SEQ ID No 4 herein below or the known plant Rb proteins in the prior art (see eg, sequences in copending WO/EP97/03070 incorporated herein by reference). Such variants preferably comprise respective parts of these amino acid
25 sequences or a sequence having at least 50% identity therewith, or still more preferably having at least 70 or at least 90% identity and most preferably at least 95% identity therewith.

 More preferably the peptide or protein comprises at least 50% of the contiguous sequence and still more preferably at least 70% thereof.

For the avoidance of doubt, particularly useful peptides or proteins for the DP function of the present invention comprise amino acids 137 to 200 of DP SEQ ID No 2, (the hetero-dimerization domain) and conservatively substituted variants thereof.

5 The peptides and proteins for use in the present method may be produced using the DNA described in the above described copending patents, particularly as expressed in whole or truncated form or as fusion with GST or MBP encoding nucleotide sequences. Useful variants of such proteins may be provided using site directed mutagenesis, eg using PCR, as is well known in the art.

10 As stated previously nucleic acid including that of SEQ ID No 1 encoding for DP of SEQ ID No 2 has been deposited on August 17th 1999 under the terms of the Budapest Treaty for the International Recognition of Microorganism Deposits for Patent Purposes of 28th April 1977 at the Coleccion Espanola de Cultivos Tipo in plasmid pCLON33 under deposit number CECT 5195. Restriction enzymes contained in the multicloning site of the plasmid can be used to excise the insert cDNA from
15 this, eg. EcoRI and XhoI or BamHI and XhoI. For in vitro transcription-translation, the full-length TmDP DNA was cloned into pBluescriptSK+ using BamHI and XhoI restriction enzymes.

A nucleic acid of SEQ ID No 3 encoding for a plant E2F of amino acid sequence of SEQ ID No 3 has been deposited on 12th May 1998 under the terms of the
20 Budapest Treaty for the International Recognition of Microorganism Deposits for Patent Purposes of 28th April 1977 at the Coleccion Espanola de Cultivos Tipo in plasmid pCLON35 under deposit number CECT5043. BamHI and XhoI, can be used to excise the insert cDNA from this. For in vitro transcription-translation, the full-length TmE2F cDNA was cloned into pBluescriptSK+ using these enzymes.

25 Suitable DNAs encoding for fusion proteins for use as binding materials of the invention provide a third aspect of the present invention.

For the purpose of using DNAs isolated and/or identified by the method of the present invention to detect similar genes in genomes other than that of plants, or even in plants other than wheat, it is possible to use computational means such as by

carrying out homology searches using eg. GENBANK or EMBL databases. Alternatively the DNAs of the genes so identified may be used as hybridization probes in low stringency and, preferably, high stringency probing of libraries of genomic DNA.

5 DNA or RNA provided from a plant or the deposit referred to above may be altered by mutagenic means such as the use of mutagenic polymerase chain reaction primers. Methods of producing the proteins or peptides of the invention characterised in that they comprise use of the DNA or RNA as described in the copending patents to express them from cells are also provided in this aspect. It may be preferred to express
10 truncates of E2F rather than whole ORFs in order to increase expression. For example, a DNA encoding a C-terminal truncate, eg lacking from 50 to 100 amino acids, particularly lacking the 80 C-terminal amino acids from SEQ ID No 4, expresses at higher level than the whole protein in E coli.

It will be seen that it may be better to express the DP hetero-dimerization
15 domain peptide or protein and E2F heterodimerization domain and DNA binding domain peptide or protein, even in labelled form, as a hetero-dimer from the same cell transformed with appropriate recombinant DNA. It may be still more preferred to express this with the Rb peptide or protein having the E2F binding domain. Such bound peptide or protein complex may be expected to express relatively well.

20 For the purpose of screening for plant DPs and E2Fs for use in the method of invention, a process which has heretofore been hampered due to human E2F and DP dissimilarity to plant E2F and DP, nucleic acid probes or primers comprising a double or single stranded DNA of sequence corresponding to 10 or more contiguous nucleotides taken from sequences SEQ ID No 1 and SEQ ID No 3 respectively are
25 provided, with the proviso that they are not selected from those just encoding for the amino acid sequence that is relatively highly conserved with human DP or E2F. Reference should be made to the aforesaid incorporated copending patents for this. Such probes and primers may be used in Northern and Southern blotting and in PCR, including RT-PCR, and LCR.

The copending patent also describe antibodies capable of specifically binding with plant E2F. Antibodies capable of binding DP factor peptides or proteins of the first aspect of the present invention are exemplified below, thus enabling the identification and isolation of further peptides and proteins of the invention and
5 nucleic acid sequences encoding therefor, eg. using techniques such as Western blotting.

The present invention will now be illustrated further by reference to the following non-limiting Examples and Figures. Further embodiments falling within the scope of the claims attached hereto will occur to those skilled in the art in the light of
10 these.

FIGURES

Figure 1 shows the sequence of TmDP (wheat DP) and the amino acid sequence encoded thereby.

15

Figure 2 shows an alignment of TmDP protein amino acid sequence with available sequences of DP proteins of animal origin. Asterisk (*) indicates amino acid identity between all sequences.

20 Figure 3 shows an alignment of partial sequences from the TmDP sequence and a deduced amino acid sequence corresponding to an Arabidopsis thaliana genomic DNA sequence of previously unknown function.

SEQUENCE LISTING

25 SEQ ID No 1 shows the nucleic acid sequence of a DNA encoding for Triticum monococcum (wheat) DP.

SEQ ID No 2 shows the amino acid sequence of Triticum monococcum (wheat) DP including leader/signal peptide sequence.

30 SEQ ID No 3 shows the nucleic acid sequence of a DNA encoding for Triticum monococcum (wheat) E2F.

SEQ ID No 4 shows the amino acid sequence of *Triticum monococcum* (wheat) E2F.
 SEQ ID Nos 5, 7, 9, 11 and 13 show the nucleic acid sequences of DNAs encoding
 for characteristic parts of the DP protein provided by the invention.
 SEQ ID Nos 6, 8, 10, 12 and 14 show the amino acid sequences of the characteristic
 5 parts of the DP protein provided by the invention.
 SEQ ID No 15 shows the nucleic acid sequence of the sense strand of double stranded
 DNA containing a canonical wild type E2F binding sequence.
 SEQ ID No 16 shows the nucleic acid sequence of the sense strand of double stranded
 DNA containing a non-binding mutant E2F canonical binding sequence.

10

GENERAL METHODS AND PROCEDURES

Experimental procedures

Wheat cell cultures

The *Triticum monococcum* suspension culture (P.M. Mullineaux; John Innes
 15 Centre, UK), was maintained as described (Xie et al., 1995).

DNA manipulations and plasmid constructions

Standard DNA manipulation techniques were applied as described (Sambrook
 et al., 1989). DNA sequencing was carried out using an Applied Biosystem 373A
 device. Oligonucleotides were from Isogen Bioscience BV (Maarsen, The
 20 Netherlands).

Plasmids pGADTmE2F, pGADTmE2F(1-373), pGADTmE2F(236-458),
 pGADTmE2F(236-373) and pGADTmE2F(391-458) were constructed as described
 previously (Ramirez-Parra et al., 1999). For *in vitro* transcription-translation, the full-
 length TmE2F and TmDP cDNAs were cloned into pBluescriptSK+ (pBS^{SK+}-
 25 TmDP). Plasmids pGADE2F-1, pGADE2F-5, pACT2-DP1 and pACT2-DP2

containing human E2F-1, E2F-5, DP1 and DP2, respectively, were provided by N. LaThangue and S. dela Luna.

Plasmid pGBT-TmE2F was made by cloning the TmE2F cDNA in frame into the pGBT8 vector (Clontech), pGBT-TmE2F(1-373) by deleting the SspI-XhoI
5 fragment of pGBT-TmE2F and pGBT-TmDP by cloning the TmDP cDNA in frame into the pGBT8 vector.

Plasmid pGEX-TmE2F(1-373) was constructed by cloning the SmaI-SspI fragment from pGADTmE2F plasmid in-frame into pGEX-KGvector (Pharmacia) and pMBP-TmDP by cloning the TmDP cDNA in frame into the pMal-c2 vector
10 (New England Biolabs). Plasmid pGFP-TmDP contains SmaI-SacI fragment from pBS^{SK+}-TmDP cloned in-frame into pAVA318 vector (von Arnim et al., 1998). Construction of wheat cDNA library and plasmid DNA preparation were carried out as described in Ramirez-Parra et al., 1999.

Production and purification of recombinant TmE2F and TmDP proteins

15 *E. coli* BL21(DE3) transformed with plasmids expressing the GST-TmE2F and MBP-DP fusion proteins were grown to an OD₆₀₀ of 0.6-0.9 and induced with 1 mM IPTG. GST-TmE2F was purified using glutathione-Sepharose beads (Pharmacia) while MBP-DP was purified using maltose agarose beads (New England Biolabs).

For the pull-down experiments, the full-length TmE2F cDNA was *in vitro*
20 transcribed and translated in the presence of ³⁵S-methionine using the TNT kit (Promega). *In vitro* binding experiments were carried out essentially as described (Huntley et al., 1998). The generation of the polyclonal serum against TmE2F using purified GST-TmE2F (236-458) has been described in Ramirez-Parra et al., 1999.

Electrophoretic mobility shift assays(EMSA)

Protein extracts for DNA binding studies were prepared essentially as described in (Bogre et al., 1997). A typical binding reaction mixture contained 20 mM Hepes, pH7.9, 12% glycerol, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM MgCl₂, 1
5 µg of salmon sperm and 10 µg of protein extract or 200ng of each bacterially purified MBP-TmDP and GST-TmE2F(1-373) proteins, as indicated. The binding mixture was incubated 20 minutes at 4°C and the DNA-protein complexes were fractionated by electrophoresis through 4% polyacrylamide gels at 4°C in 0.5xTBE buffer. Synthetic oligonucleotides indicated in the corresponding figure legend were end-
10 labeled with γ -³²P-ATP (top strand), annealed with an excess of the cold complementary bottom strand and used as a probe in the binding reactions. The same oligonucleotides without labelling were used as cold competitors. For the supershift assays, 2 µl of the polyclonal serum against TmE2F as described *ibid* were added to the binding mixture and the incubation proceeded for 10 minutes at 4°C.

Yeast two-hybrid screening and assays

Yeast growth conditions and two-hybrid analysis have been described (Fields and Song, 1989; Ramirez-Parra et al., 1999). Yeasts were first transformed with plasmid pGBT-TmE2F(1-373) and, then, with the wheat cDNA library (Xie et al., 1999; Ramirez-Parra et al., 1999). The transformation mixture was plated on yeast
20 drop-out selection media lacking tryptophan, leucine and histidine, supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT). Transformants recovered during a 3-8 days period were checked for growth in the presence of 20-30 mM 3-AT. The interaction was corroborated by α -galactosidase assay (Brecden and Nasmyth, 1985).

RNA extraction and northern blot analysis.

Total RNA from wheat cells, leaves and roots was prepared essentially as described (Xie et al., 1999). The RNA sample (10 µg) was denatured, fractionated in a 1.2% agarose gel plus 2.2 M formaldehyde, and transferred to a Zeta-Probe
5 membrane (Bio-Rad). The full-length TmDP probe was labelled by random priming with α -³²P-dCTP.

Transfection by particle bombardment and fluorescence studies.

Transient expression assays in onion epidermal cells using particle bombardment were carried out as described in von Arnim and Deng, (1994) with DNA-coated gold
10 particles (1 µm) using a Biolistic PDS-1000/He System (BioRad). Coating of gold particles was carried out essentially as described (Sanford et al., 1993; Suarez-Lopez and Gutierrez, 1997) using 2 µg of the GFP- or the GFP-TmDP-expressing plasmids per assay. After bombardment, onion inner epidermal peels were incubated as described (Varagona et al., 1992) for 16 hr at 26°C and observed under phase contrast
15 in a Zeiss Axiovert 35 microscope. GFP fluorescence was examined using 480 nm excitation light and a 510 nm long-pass filter. Phase contrast and fluorescence images were photographed, digitized and processed using Adobe Photoshop software.

EXAMPLES**20 EXAMPLE 1.****Isolation of TmDP cDNA clones**

In order to isolate protein partners for wheat TmE2F, E2F expressed from cDNA described in copending PCT/EP99/03158 was fused to the Gal4-DNA binding domain and applied to yeast two-hybrid screening. The TmE2F clone by itself trans-
25 activated the reporter genes and could not be used as bait. Therefore the last 85 amino acids of TmE2F which, based on amino acid homology studies and binding experiments, should contain the Rb-binding and trans-activation domains but not a

putative dimerization domain, were deleted. This C-terminally truncated TmE2F(1-373) does not trans-activate the reporter genes in yeast and was used as a bait in a screening of a wheat cDNA library constructed as a fusion to the Gal4-activation domain.

5 The positive clones which allowed growth of yeast colonies in selective medium (-trp, -leu, -his) were tested for growth at different concentrations of 3-AT (to detect false positive in growth) and for β -galactosidase activity. Thirty clones were isolated that grew in the presence of 30 mM 3-AT and gave a strong β -galactosidase signal. Partial DNA sequencing led us to identify and isolate 6 clones, with identical
10 DNA sequence. When we used this sequence as a query in a BLAST search against GenBank database the already identified members of the animal DP family were retrieved.

 The TmDP cDNA clone isolated by two-hybrid screen is 1089 bp long, including the poly-A tract. It contains a 5'-untranslated region and a 233 bp 3'-
15 untranslated region. The cDNA clone contains a single open reading frame of 261 amino acids (see Figure 1).

 An amino acid homology study using the CLUSTALW routine (carried out on July 25, 1999) with the available sequences of DP proteins from animal origin was carried out (Figure 2). Alignment of the TmDP with the animal DP sequences
20 available in public databases revealed the existence of several conserved motifs, strongly suggesting that the TmDP cDNA clone encodes a protein belonging to the family. This together with its ability to interact with a plant E2F protein, indicates that the TmDP cDNA encodes a bona-fide plant DP protein.

25 The CLUSTALW routine had parameters as follows

Program: : Alignment: OutputFormat: OutOrder:

ClustalW -align aln aligned ktup: 1

Window: 0 Score: TopDiag: PairGap: 0.05 Matrix: GapOpen:
 percent blosum 10

30

EndGaps: GapExt: GapDist:

10 0.05 0.05

Use JalView:

5 A search using the tfasta routine against the Arabidopsis thaliana database (carried out on July 23, 1999) did not retrieve a sequence with significant homology.

A search using the tblastn routine against the Arabidopsis thaliana database, including EST and BAC sequences (carried out on July 25, 1999) retrieves the information shown in Figure 3. This does not at first seem to correspond to an identified Arabidopsis protein as amino acids highly conserved in animal DPs are not
10 contained in the sequence retrieved.

Blast parameters were set as follows

Summary of BLAST to Arabidopsis GenBank DNA sequences using a minimum match cutoff of 50%. When many high scoring pairs (HSP) are found only a subset of the best HSPs are presented. Therefore, we can conclude that, at that date,
15 an Arabidopsis homologue of animal DP has not been identified by the genome sequencing program or by other approaches.

EXAMPLE 2.

Construction of plasmids encoding for fusion proteins.

20 Glutathione-S-transferase (GST) and Maltose binding protein (MBP) fusions are obtained as follows. Plasmid pGST-TmE2F(1-373) is conveniently constructed by cloning the fragment SmaI-SspI of TmE2F from pBS-TmE2F (pCLON35, CECT deposit #5043) in frame into the SmaI site of pGEX-KG vector (Pharmacia).

As an alternative plasmid pGST-TmE2F(1-373) was constructed by cloning
25 the fragment SmaI-NcoI of TmE2F from pGBT-TmE2F(1-373) in frame into the SmaI-NcoI sites of pGEX-KG vector (Pharmacia). pGBT-TmE2F(1-373) was constructed by cloning the fragment SmaI-SspI of TmE2F from pGAD-TmE2F(1-373) (Ramirez-Parra et al., 1999; see copending patent PCT/EP99/03158) into the SmaI site of pGBT8 vector. This is a truncated version of TmE2F lacking the C-

terminal 85 aa (the overlapping trans-activation and Rb-binding domains) but retaining the dimerization and DNA-binding domain.

Plasmid pMBP-TmDP is conveniently constructed by cloning the full-length fragment SmaI-XhoI of TmDP from pBS-TmDP (pCLON33, CECT deposit #5195) in frame into the BamHI(blunted end)-SalI sites of pMalc2 vector (New England Biolabs).

As an alternatively plasmid pMBP-TmDP was constructed by cloning the full-length fragment SmaI-XhoI of TmDP(1-261) from pGAD-TmDP (originally isolated clone in the two-hybrid screening) in frame into the BamHI (blunted end)-SalI sites of pMalc2 vector (New England Biolabs). Sequence and frame were corroborated by sequencing.

EXAMPLE 3.

Protein expression in E. coli and purification

GST-TmE2F(1-373) and MBP-TmDP were expressed in *E. coli* BL21(DE3). Transformants were grown to an OD600 of 0.6 and induced with 1 mM IPTG for 3hours at 25°C. GST and MBP fusion proteins were purified using glutathione-Sepharose beads (Pharmacia) or Amylose Resin (New England Biolabs) respectively.

Ability to specifically bind E2F responsive DNA was assessed using electrophoretic mobility shift assays (EMSA) as follows

Gel shift reactions were carried out with 50ng of GST-TmE2F(1-373) and 100ng of MBP-TmDP in binding buffer (12% glycerol, 20mM TrisHCl pH 7.8, 50mM KCl, 1mM EDTA, 1mM DTT, 1mM MgCl₂) in the presence of 1µg of salmon sperm DNA. After 15 min. of incubation (to allow hetero-dimer formation), 4 fmoles (5000 cpm) of 32P-labelled ds-oligonucleotide containing the consensus sequence for human E2F-1 binding was added for 20 minutes at 4°C. Reactions were run in 4% Acrylamide gel, 0.5X TBE buffer at 4°C at 150V to separate free and complexed DNA.

Ds-oligonucleotides: These are effectively a TmE2F binding probe and a control probe. The E2F Consensus Oligonucleotide sequences being:

E2F Wild type consensus ds-oligonucleotide

5 5' ATT TAA GTT TCG CGC CCT TTC TCA A 3' E2F WT-Sense
3' TAA ATT CAA AGC GCG GGA AAG AGT T 5' E2F WT-Antisense

E2F Mutant ds-oligonucleotide (non-binding control)

5' ATT TAA GTT TCG ATC CCT TTC TCA A 3' E2F Mut-Sense
10 3' TAA ATT CAA AGC TAG GGA AAG AGT T 5' E2F Mut-Antisense

The binding sites are underlined. The sense oligonucleotide was 32P-labelled and then annealed to a sufficient excess of cold antisense oligonucleotide to ensure that labelled, free oligonucleotide is undetectable.

15

EXAMPLE 4.

Preparation of column capable of specifically binding transcription factor target DNA.

Glutathione SepharoseTM 4B is obtained as Catalog # 17-0756-01 (Amersham Pharmacia Biotech AB). Amylose resin is obtained as Catalog # 800-215 (New England Biolabs). Buffer conditions are as used for EMSA binding buffer with the modification that glycerol concentration may be reduced to 6% if desired. ie. 6% glycerol, 20mM TrisHCl pH 7.8, 50mM KCl, 1mM EDTA, 1mM DTT, 1mM MgCl₂.

The GST-E2F-containing bacterial protein extract is applied to the Glutathione Sepharose resin, according to the manufacturer's instructions using the buffer described above. While MBP-DP-containing bacterial protein extract is applied to the Amylose resin, according to the manufacturer's instructions, using that same buffer. Proteins are eluted as pure according to manufacturer's instructions and equimolar amounts of both are mixed at 4°C for 1 hour.

The mixture is applied through a new Glutathione Sepharose resin, equilibrated in the same buffer to purify the hetero-dimer, after washing according to

the manufacturer's instructions. NB: the mixture can be passed through an amylose resin to retain the hetero-dimer via the MBP moiety. The hetero-dimer bound to the resin is now ready to be used to retain the pretreated genomic DNA. Pretreatment of this DNA is by shearing or by digestion with Sau3A to provide fragments.

5

EXAMPLE 5**Purified plant E2F binds DNA with arelatively low efficiency**

In order to study in more detail E2F-DNA protein complex formation we carried out EMSA with the bacterially-expressed and purified GST-TmE2F fusion protein which contained the deletion of the last 85 amino acids, but still retained the conserved DNA-binding domain (this truncated version was expressed in much larger amounts than the full-length protein).

10

A single DNA-protein complex was detected with the wild type E2 binding site probe, when increasing amounts of purified TmE2F were added to the binding mixture. The formation of this TmE2F-DNA complex was specific since (i) complex formation depends on an intact E2F binding site as it did not occur when the E2 mutant probe, containing the point mutations within the canonical E2F binding site, was used, and (ii) when purified GST proteins was added. To confirm these observations we also carried out competition experiments. Adding increasing molar excessof the E2 wild type probe, but not of the mutated E2 mutant probe, was able to compete out the preformed TmE2F-DNA complexes. Altogether, these data indicate that TmE2F binds effectively to one of the DNA sequences described as a canonical E2F DNA-binding sequence for human E2F-1 and that this binding was specific and depends on an intact E2F binding site.

15

20

It should be pointed out, however, that in these binding studies the amount of purified E2F protein needed to efficiently form a complex was relatively high, suggesting that complex formation was not optimal, even under a wide variety of binding conditions (not shown).

25

EXAMPLE 6.

Heterodimerization properties of TmE2F and TmDP

It has been previously shown that human DP-1 and DP-2 can form stable heterodimer with any of the E2F family members (Bandara et al., 1993; Helinet al., 1993; Sardet et al., 1995; Ormondroyd et al., 1995), a situation which is different from the more
5 stringent interaction observed between E2F and pocket proteins (Dyson, 1998; Ramirez-Parra et al., 1999). To investigate the heterodimerization properties of TmDP the inventors used a yeast two-hybrid approach using E2F and DP proteins of different sources. All the combinations tested between human (HuE2F-1, HuE2F-5, HuDP-1 and HuDP-2) and plant (TmE2F and TmDP) proteins allowed efficient
10 growth of the cotransformant in selective medium, supplemented with 20 mM 3-AT and were positive in the β -galactosidase assay. Therefore, we can conclude that the protein domains involved in heterodimerization are functionally conserved between organisms as divergent as human and wheat, a result consistent with the high amino acid conservation observed in their heterodimerization domains. Deletion
15 experiments confirmed that a central domain in TmE2F, which contains the leucine zipper, is necessary and sufficient to mediate the heterodimerization with TmDP.

EXAMPLE 7.

TmDP stimulates binding of TmE2F to a canonical DNA binding site

To determine whether TmDP had any functional effect on the TmE2F DNA
20 binding activity, the inventors carried out EMSA with purified proteins. Addition of purified MBP-TmDP to a DNA probe containing a canonical E2F binding site (E2 wild type oligo) did not produce any retarded band, indicating that TmDP alone does not bind to DNA. However, under conditions of low amounts of TmE2F, where DNA binding was virtually undetectable, adding increasing amounts of purified
25 MBP-TmDP very significantly stimulated complex formation. Therefore, it can be

concluded that heterodimerization of TmE2F with TmDP contributes to a several-fold increase in the affinity and/or stability of the DNA-protein complex.

EXAMPLE 8.

5 Demonstration that TmDP is a cytoplasmic protein

To investigate the subcellular localization of TmDP we used transient expression assays after biolistic delivery to onion epidermal cells (Varagona et al., 1992) of a translational fusion of TmDP to the greenfluorescent protein (GFP; Sheen et al., 1995) under the control of the CaMV 35S promoter, which allowed us to
10 transiently follow the expression of the chimeric construct. In all cases observed, the TmDP-GFP fusion protein was present in the cytoplasm of transfected onion cells and apparently excluded from the nucleus, a pattern which was different from that of the control cells expressing GFP alone which is known to also diffuse to the nucleus. This observation is consistent with the lack of a nuclear localization signal (NLS) in
15 the TmDP amino acid sequence. Therefore, it is most likely that the putative NLS identified in TmE2F is responsible for actively transporting the TmDP-TmE2F heterodimer to the nucleus.

To demonstrate that TmDP alone does not have transactivation potential in yeast the inventors transformed yeast cells with a plasmid expressing TmDP fused to
20 the Gal4 DNA-binding domain and plated them under selective conditions (-trp, ±his) in the presence of 20 mM 3-AT. Yeast cells were unable to grow under those conditions, as they were those carrying the vector alone, while yeast cells expressing TmE2F fused to the Gal4 DNA binding domain could grow, indicating that TmDP lacks transactivation potential on its own. In the same assay, human DP-1 and DP-2
25 alone do not transactivate either. Therefore, it is likely that the transactivation ability of the TmE2F/TmDP heterodimer is conferred by the C-terminal domain of TmE2F

in the E2F/DP heterodimer, although the presence of the DP partner may cooperate in E2F transactivation by stabilizing the DNA-protein complex.

EXAMPLE 9.

Characterisation of TmDP

5 The idea that the isolated cDNA encodes a plant member of the DP family was reinforced by analysis of the amino acid homology and domain organization. TmDP exhibits an overall 29-33% amino acid similarity with human (Bandara et al., 1993; Girling et al., 1993; Krek et al., 1993) and *X. laevis* (Girling et al., 1994) DP-1 and DP-2 and a slightly smaller similarity (27%) with *D. melanogaster* DP (Dynlacht
10 et al., 1994; Ohtani and Nevins, 1994). Amino acid alignment of plant and animal DP proteins indicates that it has a similar domain organization (Fig. 3B). The highest homology occurs within a 70 amino acid region (residues 64-143 in TmDP) which in animal DP proteins are important for DNA binding (Wu et al., 1996). This region includes a 10 amino acid stretch of fully conserved residues. Other amino acid blocks
15 with a significant degree of homology contain the heptad repeats (residues 144-213 in TmDP), involved in heterodimerization with E2F (Wu et al., 1996; Zheng et al., 1999) and the domain conserved with E2F proteins (residues 214-240 in TmDP), a region which is similar to the E2F family members (Girling et al., 1993). Experimental evidences of the heterodimerization properties of TmDP will be
20 presented below in detail. Quite interestingly, TmDP lacks an acidic region which is present near the C-terminus of animal DP members, a domain whose functional significance has not been determined. Finally, the less conserved region corresponds to the N-terminal domain whose length and amino acid sequence is similar to that of animal DP members, in particular to the DP-2 group. Based on these homology
25 studies, we conclude that TmDP presents a higher amino acid sequence similarity to animal DP-2. However, it is worth noting its smaller size and the absence of an acidic C-terminal domain as unique properties of TmDP.

To confirm that the cDNA isolated contained a single ORF *in vitro* transcription and translation reactions of a suitable construct were carried out in the

presence of ^{35}S -labelled methionine. A major ~ 30 kDa band was obtained, consistent with the predicted molecular mass. Finally, to determine whether TmE2F/TmDP interaction can occur in the absence of other proteins, we carried out *in vitro* pull-down experiments. When *in vitro* translated ^{35}S -labelled TmE2F was mixed with
5 bacterially expressed and purified MBP-TmDP, but not with MBP alone, a significant amount of the input material remained bound to the resin indicative of a direct interaction between TmE2F and TmDP.

Northern analysis of total RNA samples reveals the existence of a major $\sim 1.9 \pm 0.2$ kb transcript together with another much larger transcript (3.5 ± 0.2 kb).
10 Both transcripts were present in cultured cells, where they were more abundant, as well as in roots and leaves. However, the smaller transcript was more abundant in leaves. The larger transcript may represent a partially processed RNA species or the result of an alternative splicing. It is important to mention that alternative splicing is characteristic of mammalian DPproteins (Ormondroyd et al., 1995; de la Luna et al.,
15 1996; Rogers et al., 1996; Wu et al., 1996), although its functional significance has not been established yet.

The murine homolog of human DP-2 (DP-3) is unique in that its primary transcript undergoes extensive alternative splicing giving rise to a final complex mixture of four products (α , β , γ and δ ; Ormondroyd et al., 1995). The α and δ
20 proteins share a common alternative spliced exon, encoding 16 amino acid residues, known as the E region, which is absent in the β and γ isoforms as well as in DP-1 and which seems to function as a nuclear localization signal (NLS; de la Luna et al., 1996). The β , γ and δ isoforms are produced after initiation of translation in a methionine residue downstream from the first methionine, used in the α isoform. In
25 addition, the γ isoform has an extra glutamine residue. Thus, based on the amino acid sequence of TmDP, the presence of a relatively short N-terminal region, the lack of an apparent E region, and consequently of a NLS, and the lack of the Q residue within the DNA-binding domain, TmDP seems to be structurally related to the murine DP-2 β isoform (de la Luna et al., 1996). It is interesting to note that the small size of

TmDP does not seem to be caused by a lack of N-terminal residues but, rather, to a lack of a large C-terminal region, which is present in animal DP proteins. In the latter, this region is highly acidic but its function has not been established yet (Wu et al., 1995; Zhang and Chellapan, 1995).

- 5 While this work was in process, the sequence of genomic regions of *A.thaliana* encoding putative DP-like proteins has been released. In the absence, sofar, of sequence information of the corresponding cDNAs, it is difficult to make a detailed homology study due to potential uncertainties inherent to the amino acid predictions of the intron/exon sequences. An additional complication in the case of
- 10 DP-like proteins may derive from the possibility of differentially spliced forms of plant DP transcripts. As in the case of TmDP, the predicted sequences of *A. thaliana* DP-like proteins have a relatively short N-terminus and lack the extended C-terminal end characteristic of the animal DP proteins.

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CLAIMS

1. A method of controlling one or more of plant growth, gene expression, cellular DNA replication, cell cycle progression, differentiation and development comprising
5 increasing or decreasing E2F-dimerization partner (DP) protein activity in a plant cell through expression of a recombinant DP peptide or protein in that cell characterised in that the peptide or protein comprises a sequence SEQ ID No 2, a functional part thereof, or a sequence having at least 70% homology to either, that peptide or protein being capable of interacting with a plant E2F protein or peptide such as to alter E2F
10 activity in the plant cell.
2. A method as claimed in Claim 1 characterised in that the peptide or protein sequence is of 50% or more identity with that of the corresponding full length or part of SEQ ID No 2.
15
3. A method as claimed in Claim 1 or Claim 2 characterised in that the peptide or protein sequence is of 70% or more identity with that of the corresponding full length or part of SEQ ID No 2.
- 20 4. A method as claimed in any one of the preceding claims characterised in that the plant DP activity comprises one or both of (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate E2F binding to E2F/DP transcription factor binding sites in plant DNA.
- 25 5. A method as claimed in any one of the preceding claims characterised in that it includes steps of altering the plant DP protein level, the E2F-DP DNA-binding activity, transactivation properties, and/or the DP/E2F-binding activity.

6. A method as claimed in any one of the preceding claims characterised in that the DP may be modified alone and/or in combination with a modification of the levels or activity of plant E2F and/or plant Rb.

5 7. An isolated, enriched, cell free and/or recombinantly produced protein or peptide, capable of altering E2F-dimerization partner (DP) activity in a plant cell, characterised in that it has one or both DP activities in plants selected from (i) the ability to dimerize with plant E2F protein and (ii) the ability to modulate, particularly enhance, E2F binding to E2F transcription factor binding sites in plant DNA or effect
10 thereof

characterised in that the protein or peptide comprises an amino acid as shown in SEQ ID No 2 or a functionally active part thereof or a sequence having at least 70% homology to such sequence or part.

15 8. A protein or peptide as claimed in Claim 7 characterised in that it has at least 50% identity to the amino acid sequence as shown in SEQ ID No 2 or a functionall active part thereof.

9. A protein or peptide as claimed in Claim 7 or Claim 8 characterised in that it is
20 of SEQ ID No 2 or is a variants thereof modified such that the amino acid sequence is mutated such that its ability to dimerize with E2F protein is reduced from that of the native sequence or abolished completely therefrom, whereby the peptide is capable of acting as a DP protein which decreases or abolishes native or recombinant E2F binding to its DNA binding site, thus inhibiting or abolishing E2F activity in a cell in
25 which is its present.

10. A protein or peptide as claimed in any one of the preceding Claims 7 to 9 characterised in it comprises a sequence found in that of SEQ ID No 2 or having at
30 least 70% homology thereto selected from those comprising

SEQ ID No 6 ARAAMAPPRGGAAAAATAALDLTGvhILEAS SVpPLPE
RGGNAVQRKGAVDP

SEQ ID No 8 DKDRKKEKAAAPRITGWGLREYSKIVCEKVEAKGRT TY
NEVADEIYSELKS

5 SEQ ID No 10 MAHIGQGfDEKNIRRRVYDAFNVLIALRVIAKEKKEIR
W MGLSNRYEYKIKKLEEV

SEQ ID No 12 RKELVNKIRNKKALLQEIEKQFDDLQNIKLRNQTLESS A
ENVNGIRLPFVLVKTSR

10 SEQ ID No 14 KARVEIEISDDSKFAHFENGAPFTLHDDLSILEGVRGNS
IGKAGRATLH

11. A protein or peptide as Claimed in Claim 10 characterised in that the sequence
comprises two or more of these sequences or sequences at least 70% homologous
thereto.

15

12. Isolated, enriched, cell free and/or recombinant nucleic acid comprising a
sequence encoding for expression of a protein or peptide as described in any one of
Claims 7 to 11.

20

13. Nucleic acid as claimed in Claim 12 characterised in that it includes the
coding nucleic acid sequence of SEQ ID No 1 or a part thereof encoding for all or a
functional part of the amino acid sequence shown therein as defined above.

25

14. Nucleic acid as claimed in Claim 12 or Claim 13 as contained in plasmid
pCLON33, deposit number CECT 5195 made on August 17th 1999 under the terms of
the Budapest Treaty for the International Recognition of Microorganism Deposits for
Patent Purposes of 28th April 1977 at the Coleccion Espanola de Cultivos Tipo.

15. A nucleic acids as claimed in any one of Claims 12 to 14 characterised in that it encodes for a plant DP or a functional variant thereof including the sequence of SEQ ID No 1 or a sequence complementary thereto or otherwise antisense thereto.
- 5 16. A nucleic acid probe characterised in that it comprises a DNA sequence corresponding to an amino acid sequence selected from SEQ ID No 2 to 8.
17. A nucleic acid probe or primer characterised in that it comprises a double or single stranded DNA of sequence corresponding to 10 or more contiguous nucleotides
10 taken from the sequence SEQ ID No 1 with the proviso that it is not selected from amino acids 70 to 136. Such probes and primers may be used in Northern and Southern blotting and in PCR, including RT-PCR, and LCR.
18. An oligonucleotides probe characterised in that it comprises at least 18
15 contiguous bases of the SEQ ID No 1.
19. An oligonucleotide probe as claimed in Claim 18 characterised in that it is 30 to 100 bases long.
- 20 20. An oligonucleotide primer as claimed in Claim 17 characterised in that it is of 10 to 20 bases long.
21. Antisense DNA to a nucleic acids as claimed in any one of Claims 12 to 20.
- 25 22. A nucleic acid characterised in that it encodes a the DP protein or peptide encoding sequence as claimed in any one of Claims 7 to 11 together with a sequence encoding an E2F protein or peptide.

23. A nucleic acid as claimed in Claim 22 characterised in that the sequences encoding the DP and E2F encoding sequences are under control of the same regulatory element or elements.

5 24. A nucleic acid vector or construct comprising a nucleic acid as claimed in any one of Claims 12 to 23 or antisense nucleic acid thereto.

25. A plant cell comprising recombinant nucleic acid, a vector or a construct as claimed in any one of Claims 12 to 24.

10

26. A transgenic plant or part thereof comprising recombinant nucleic acid, a vector, DNA construct or cell as claimed in any one of Claims 12 to 25.

15 27. An antibody characterised in that it has been raised against a DP peptide or protein as claimed in any one of Claims 7 to 11.

28. An antibody as claimed in Claim 27 characterised in that it is capable of specifically binding with plant DP factor peptides or proteins as claimed in any one of Claims 7 to 11, but not to the human, mouse or *Xenopus* DP.

20

29. A method for identifying and/or isolating DNAs corresponding to complete or partial genes that are regulated in G1 passage, G1/S-phase transition and/or S phase progression of the cell cycle, said method comprising contacting a sample of genomic DNA with a binding material specific for binding such complete or partial genes, removing non-bound DNA from the specific binding material then, releasing and isolating the bound DNA.

25 characterised in that the specific binding material comprises a peptide or protein including the DNA binding sequence of a protein that is capable of acting as a part of a plant hetero-oligomer transcription activator or repressor.

30

30. A method as claimed in Claim 29 characterised in that the specific binding material comprises a peptide or protein which includes a plant E2F DNA binding domain.
- 5 31. A method as claimed in Claim 30 characterised in that the specific binding material comprises a peptide or protein which includes a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) hetero-dimerization domain and/or a plant retinoblastoma protein E2F binding domain.
- 10 32. A method as claimed in any one of Claims 29 to 31 characterised in that the material comprises a peptide or protein that comprises a plant E2F DNA binding domain together with a plant E2F-dimerization partner (DP) binding domain together with a peptide or protein that includes a plant dimerization partner (DP) E2F binding domain.
- 15 33. A method as claimed in any one of Claims 29 to 32 characterised in that the E2F and DP domains are comprised in *Triticum monnococtum* sequences or functional variants or parts thereof.
- 20 34. A method as claimed in Claim 33 characterised in that the DP domains are comprised in peptides or proteins as claimed in any one of Claims 7 to 11.
35. A method as claimed in any one of Claims 29 to 34 characterised in that the specific binding material peptide or protein is labelled or tagged to assist in
25 identifying or immobilising it, particularly when in bound complex with the gene to be identified and/or isolated.
36. A method as claimed in any one of Claims 29 to 35 characterised in that the specific binding material comprises two peptides or proteins, one including the E2F

DNA binding domain, and one including the DP hetero-dimerization domain bound together as a hetero-dimer.

37. A method as claimed in any one of Claims 29 to 36 characterised in that the
5 E2F sequence is all or part of SEQ ID No 4 or a sequence having 90% homology thereto.

38. A method as claimed in any one of Claims 29 to 37 characterised in that the
binding material comprises a peptide or protein including an optionally labelled plant
10 retinoblastoma protein E2F binding domain.

39. A specific binding material characterised in that it comprises a peptide or
protein having DNA binding activity with respect to plant DNA transcription
activator or repressor factor binding sites, particularly in genomic DNA, and having
15 the ability to dimerize or oligomerize with a further such plant protein together with one or more of said further a peptides or proteins.

40. A specific binding material characterised in that it comprises a peptide or
protein having DNA binding activity with respect to plant DNA E2F transcription
20 factor binding sites, particularly those present in genomic DNA and having the ability to dimerize with plant DP protein together with one or both of

(ii) a peptide or protein that is capable of binding to plant E2F through its DP
hetero-dimerization domain and

(iii) a peptide or protein that is capable of binding to plant E2F through its
25 retinoblastoma protein binding domain.

41. A material as claimed in Claim 40 characterised in that the peptides or
proteins are wheat peptides or proteins and functional variants and parts thereof.

42. A material as claimed in Claim 40 or 41 characterised in that it comprises a hetero-dimer of (i) and (ii) or (i) and (iii).
43. A material as claimed in any one of Claims 40 to 42 characterised in that it
5 comprises both of the peptides or proteins (i) and (ii) in the form of a hetero-dimer together with peptide or protein (iii).
44. A material as claimed in any one of Claims 40 to 43 characterised in that the
10 peptides or proteins are in labelled or tagged form.
45. A material as claimed in any one of Claims 40 to 44 characterised in that it is
in the form of a coating or otherwise bound form on a support material.
46. A material as claimed in any one of Claims 40 to 44 characterised in that the
15 material is in the form of particles of the peptides or proteins and/or hetero-dimers or trimers.
47. A nucleic acid characterised in that it encodes for a peptide or protein as
claimed in any one of Claims 7 to 11 fused to a sequence encoding for a protein label.

20

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BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE,
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IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT,
LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ,
PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT,
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
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CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: WHEAT DP PROTEINS AND USES THEREOF

```

1GAATTCGGCAGCGCGCAATGGCGCCTCCCGCGGCGGAGCTGCTGCGGCGGCTACCGCC 61
1M A P P R G G A A A A A T A 14
61GCACTGGACCTGACCGCGTGCACATTCTCGAAGCTTCCAGTGTCCCGCGCTTCCCGAA 120
15A L D L T G V H I L E A S S V P P L P E 34
121CGCGCGGTAAATCGGTCCAAAGGAAGGGGCTGTGACCGGATAAGATAGGAAGAAG 180
35R G G N A V O R K G A V D P D K D R K K 54
181GAGAAGGCTCGGCGACCGGAGGATCACCGGTGGGGGCTCGCGAGTACAGCAAAATAGTT 240
55E K A A A P R I T G W G L R E Y S K I V 74
241TGTGAGAAAGTTGAAGCCAAAGGAACAACATACATGAGGTTGACAGCAAAATTTAT 300
75C E K V E A K G R T T Y N E V A D E I Y 94
301TCAGAGCTGAAGTCCATGGCACATATTGGTCAAGGGTTTGATGAGAAGATATTAGCGG 360
95S E L K S M A H I G Q G F D E K N I R R 114
361AGAGTGATGATGCTTTCAACGTTCTCATTGCACCTCGTGTATTGCAAAAGAAAAAG 420
115R V Y D A P N V L I A L R V I A K E K K 134
421GAGTACGGTGGATGGGCTTTCAAATTACAGATATGAAAAATAAAGAACTTGAGGAA 480
135E I R W M G L S N Y R Y E K I K K L E E 154
481GTTCTAAAGAACTCGTCAACAAGATTAGGAACAAGAAGGCACTCTCCAGGAAATCGAA 540
155V R K E L V N K I R N K K A L L Q E I E 174
541AAACAGTTTGATGATCTCCAAAACATCAAGTTACGTAAACCAACACTGGAAAGCTCAGCA 600
175K Q F D D L Q N I K L R N Q T L E S S A 194
601GAGAATGTTAATGGCATCCGCTTCCATTGCTATTGGTCAAGACATCTAGGAAAGCAAG 660
195E N V N G I R L P F V L V K T S R K A R 214
661GTGAAATTTAGATTTCAGATGACTCGAAGTTTGCCATTTCGAGTTCAATGGTGACCA 720
215V E I E I S D D S K F A H F E F N G A P 234
721TTCACATTGCATGATCTCTCAATCCTTGAGGGGTAAAGCGTAACAGCATAGGAAGA 780
235F T L H D D L S I L E G V R R N S I G R 254
781CTGCGCGCGCCACCTTCACTAGAGACTCAAGAATATTACAAATGAATTAAGTGTTA 840
255A G R A T L H *261
841GAACTGCACAGCGGATTCTTTGACAGCTATGTATAGCTATATATCCTCATGAAAAC 900
901TTGACCTAGTTTATAGACAGTCTCTCAGGCTTGAGAAGATTTTAACCTGCAAAATTTGT 960
961CTCCTTTTGTGCTAGCAGGTTATAGGTCTCAGATAGATGATTCATATATGTGCTGCT 1020
1021ATGAAAACATTGTAGCAAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAA 1080
1081AAAAAA1089

```

Sequence of TmDP cDNA and deduced amino acid sequence.

(57) Abstract: A method of controlling plant cell cycle is provided characterised in that it comprises increasing or decreasing E2F-dimerization partner (DP) protein activity in a plant cell through expression of a recombinant DP peptide or protein in that cell. Further provided is use of such proteins in identifying genes involved in cell cycle control.

WO 01/21644 A2

1GAATTGGCAGAGCCGCAATGGCGCCTCCCCGGCGGAGCTGCTGGGCGCGTACCGCC 61
1 M A P P R G G A A A A A T A 14
61 GCACTGGACCTGACCGCGTGCACATTCCTGAAGCTTCCAGTGTCCCCCGCTTCCCGAA 120
15 A L D L T G V H I L E A S S V P P L P E 34
121 CGCGCGGTAAATGCGGTCCAAAGGAAGGGGCTGTGACCCCGGATAAAGATAGGAAGAAG 180
35 R G G N A V Q R K G A V D P D K D R K K 54
181 GAGAAGGCTGCGGCACCGAGGATCACCGGTGGGGCTCGCGAGTACAGCAAAATAGTT 240
55 E K A A A P R I T G W G L R E Y S K I V 74
241 TGTGAGAAAGTTGAAGCCAAAGGAAGAACACATACAATGAGTTGCAGACGAAATTTAT 300
75 C E K V E A K G R T T Y N E V A D E I Y 94
301 TCAGAGCTGAAGTCCATGGCACATATTGGTCAAGGGTTTGATGAGAAGAATATTAGCGG 360
95 S E L K S M A H I G Q G F D E K N I R R 114
361 AGAGTGTATGATGCTTTCAACGTTCTCATGTGCACCTCGTGTATTGCAAAAGAAAAAAG 420
115 R V Y D A F N V L I A L R V I A K E K K 134
421 GAGATACGGTGGATGGCCCTTCAAAATTACAGATATGAAAAATAAAGAGCTTGAGGAA 480
135 E I R W M G L S N Y R Y E K I K L E E 154
481 GTTCGTAAGAACTCGTCAACAAGATTAGGAACAAGAGCAGCTCTCCAGGAATCGAA 540
155 V R K E L V N K I R N K K A L L Q E I E 174
541 AAACAGTTTGATGATCTCCAAACATCAAGTTACGTAAACCAACACTGGAAAGCTCAGCA 600
175 K Q F D D L Q N I K L R N Q T L E S S A 194
601 GAGATGTTAATGGCATCCGCTTCCATTCGTATTGGTCAAGACATCTAGGAAAGCAAGG 660
195 E N V N G I R L P F V L V K T S R K A R 214
661 GTGGAAATTGAGATTTGAGATGACTCGAAGTTTGCCCATTTGAGTTCAATGGTGCACCA 720
215 V E I E I S D D S K F A H F E F N G A P 234
721 TTCACATTGCATGATCTCTCAATCCTTGAGGGGTAAGCGTAACAGCATAGGAAGA 780
235 F T L H D D L S I L E G V R R N S I G R 254
781 GCTGGCCGCGCCACCTTCACTAGAGACTCAAGAATATTACAAATGAATTAAAGTGTTA 840
255 A G R A T L H *261
841 GAACTGGCACAGCCGGATTCCTTTGCACAGCTATGTATAGCTATATATCCTCATGAAAC 900
901 TTGACCTAGTTTATAGGACAGTCTCTCAGGCTTGAGAAGATTTTAACCTGCAAAATTTGT 960
961 CTCCTTTTGTGCTAGCAGGTTATTAGGCTCAGATAGATGATGATGATGATGCTGCT 1020
1021 ATGAAAAACATTGATAGCAAAAAAATAAATAAATAAATAAATAAATAAATAAATAA 1080
1081 AAAAAAAAA1089

FIG. 1 Sequence of TmDP cDNA and deduced amino acid sequence.

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```

hDP-1  MAKDAGLIEANGELKVFIDQNLSPGKGVVSLVAVHPSTVNPGLKQLLPKTFGQSNVNIQA 60
mDP-1  MAKDASLIEANGELKVFIDQNLSPGKGVVSLVAVHPSTVNTLGKQLLPKTFGQSNVNIQA 60
X1DP-1 MAKDAGLIEANGELKVFVDQNLSPGKGVVSLLTVHPSSISSLGKQLLPKTFGQSTVNISQ 60
hDP-2  -----
mDP-2  -----
X1DP-2 -----
DDP    -----RLQDNGLSIPKTEAGT 16
TmDP   -----NS 2

hDP-1  QVVIGTPORPAASNT-----LVVGS---PHTP-STHFASQNQPSDSSPWSAG----- 103
mDP-1  QVVIGTPORPAASNT-----IVVGS---PHTP-NTHFVSQNQTSDDSPWSAG----- 103
X1DP-1 QVVLGTPORQAPNT-----ILIGS---PHTP-NTHFVSQNQATDSSPWSAG----- 103
hDP-2  -MIISTPQRLTSSGS-----VLIGS---PYTP-APAMVTQTHIAEATGWVPGDRKRARKF 50
mDP-2  -MIISTPQRIANSGS-----VLIGN---PYTP-APAMVTQTHIAEAGWVP----- 41
X1DP-2 -MIISTAQRLSVAGD-----LLIGS---SYAANTSAMVTQSHITEATWI PGDRKRAREF 51
DDP    TYTTVSAQKTSAGSGHYDLPKGDYVKEFTPNPIKMKSKLHAIQSNLSHMS---ASS- 72
TmDP   ARAAMAPPRGGAAAATAALDLTGVIHILEASSVPPPLPERGGNAVQRKGAVDP----- 54
      :. : . : * : : :

```

FIG. 2 Amino acid alignment of TmDP protein with available sequences of DP proteins from animal origin. * = identical amino acid.

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hDP-1  KTVIDCSISNDKFEYLFNFDNT-FEIHDDIEVLKRMGMACGLESGCSAEDLKMARSLVP 327
mDP-1  KTVIDCSISNDKFEYLFNFDNT-FEIHDDIEVLKRMGMACGLESGNCSAEDLKVARSLVP 327
x1DP-1 KTVIDCSISNDKFEYLFNFDNT-FEIHDDIEVLKRMGMACGLESGCSAEDLKTAKS LVP 328
hDP-2  KTVIDCSISSDKFEYLFNFDNT-FEIHDDIEVLKRMGMSFGLSGKCSLEDLKLAKSLVP 281
mDP-2  KTVIDCSISSDKFEYLFNFDNT-FEIHDDIEVLKRMGMSFGLSGKCSLEDLKLAKSLVP 266
x1DP-2 RTVIDCSISSDKFEYLFNFDNA-FEIHDDIEVLKRMGMSFGLSGKCSLEDLKLAKSFVP 282
DDP     STKINCSVTNDKSEYIFKFDKT-FEMLDDIEVLKRMGFLGLDKGECTPENIERVKS WVP 294
TmDP    KARVEIEISDDSKFAHFEFNGAPFTLHDDL SILEGVRGNSIGKAGRATLH----- 267
      : :: : : : * : : * : : * : : * : : * : : * : : * : : * : : * : :
hDP-1  KALEPYVTEMAQGT--VGGVFITT-AGSTSNGRFS-----ASDLTNGADGMLAT- 374
mDP-1  KALEPYVTEMAQGS--IGGVFVTT-TGSTSNGRFLS-----ASDLTNGADGMLAT- 374
x1DP-1 KALEPYVTEMAQGS--ISSVYISFSSGSVSNRRFS-----SSDLTGCTDGM LAT- 376
hDP-2  KALEGYITDISTGPSWLNQGLLLNSTQSVSNLDLTGATLPQSSVNQGLCLDAEVALATG 341
mDP-2  KALEGYITDISTGPSWLNQGLLLNSTQSVSNLDPTTGATVPQSSVNQGLCLDAEVALATG 326
x1DP-2 RALEGYVTEMATGSSWADQSFNSQTVSSAGNTTCTT--TNSQASLTSGLYFDSEVSLTT- 339
DDP     PNLAKYVEAYGTGKT-GENMYESDDEDFNFGYLES-----ANESQGF AQH- 339
TmDP-----

hDP-1  -----SSNGSQYSGSRVETPVSVYGEDDEEDDDFNENDEED 410
mDP-1  -----SSNGSQYSGSRVETPVSVYGEDDDDDDDFNENDEED 410
x1DP-1 -----SSNGSQYSSSRVETPVSVYEEEDDDDDDDDLDDD-- 409
hDP-2  QFLAPNSHQSSSAASHCSESERGETPCSFNDEDEDEEDSSSPE-- 385
mDP-2  QLPASNHQSSSAASHFSESERGETPCSFNDEDEDEEDSSSPE-- 370
x1DP-2 -----SSHSSSGTSHYTESERGETPCWFDDDD--EDDEDDSSSME-- 376
DDP     -----S-AQHTTD--GEFKLEMDDDELDDID----- 363
TmDP-----

```

FIG. 2 (continued)

5/5

Score = 79 (27.8 bits), Expect = 9.5, P = 1.0
 Identities = 46/188 (24%), Positives = 98/188 (52%), Frame = +3

Query: 64 GW--GLREYSKIVCEKVEAKGRRTTYNEVADEIYSELKSMAHIGQGFDEKNIR-RRVYDAF120
 GW R S+ V +VEA G + + +E+ +K ++ GF EK++ ++ + +

Sbjct: 75510 GWYNESRRDSETVKARVEA-GLSEVKKSVEELALLIKR-SNRSAGFQEKDMEVLKMEEKY75683

Query: 121 -NVLIALRVIAKEKKEIRWMGLSNRYEKI---KKLEEVVKELVNKIRNKKALLQEIEXQ176
 V+ L V+ +E ++ + +S+ E++ +K+EE+R + +R ++L +EIE

Sbjct: 75684 AEVMRVLEVVKKEEVSrvK-LDVSSVLIERVAAEEKVEELRFKTEGGLRLLLESLLKKEIEVA75860

Query: 177 FDD-----LQNIK-LRN-QTLESSAENVNGIRLPFVLVKTsrKARVEIEISDDSKFAHFE229
 ++ L I+ L+ + +E E I++ +LV+ +++ + +E ++ SK E

Sbjct: 75861 NEEHLMVALGKIEALKGYKEIERQREG-KAIKVLDLlVERNKRlKNMLEEAEERSKDIEIE76037

Query: 230 FNGAPFTLHDDLSILE 245
 F D+ +LE

Sbjct: 76038 L-----FETSTDVEMLE 76073

FIG. 3

CSIC - OTI
000284 03.06.02
ENTRADA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**Crisanto GUTIERREZ-ARMENTA and
Elena RAMIREZ-PARRA**

PCT/EP00/09325

25 September 2000

For: WHEAT DP PROTEINS AND USES THEREOF

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Barbara L. Mullin	Registration No. <u>38,250</u>
Michael P. Straher	Registration No. <u>38,325</u>
Kevin M. Flannery	Registration No. <u>35,871</u>
David A. Cherry	Registration No. <u>35,099</u>
Lynn A. Malinoski	Registration No. <u>38,788</u>
Steven B. Samuels	Registration No. <u>37,711</u>
Janet E. Reed	Registration No. <u>36,252</u>
Jeffrey J. King	Registration No. <u>38,515</u>
Robert B. Washburn	Registration No. <u>16,574</u>
Norman L. Norris	Registration No. <u>24,196</u>
Michael J. Swope	Registration No. <u>38,041</u>
Michael J. Bonella	Registration No. <u>41,628</u>
Harold H. Fullmer	Registration No. <u>42,560</u>
John E. McGlynn	Registration No. <u>42,863</u>
Jonathan M. Waldman	Registration No. <u>40,861</u>
Chad Ziegler	Registration No. <u>44,273</u>
Gwilym J.O. Attwell	Registration No. <u>45,449</u>
David N. Farsiou	Registration No. <u>44,104</u>
Paul K. Legaard	Registration No. <u>38,534</u>
Steven H. Meyer	Registration No. <u>37,189</u>
Paul B. Milcetic	Registration No. <u>46,261</u>
Joseph R. Condo	Registration No. <u>42,431</u>
Michael K. Jones	Registration No. <u>41,100</u>
Frank T. Carroll	Registration No. <u>42,392</u>
Thomas E. Watson	Registration No. <u>43,243</u>
Peter M. Ullman	Registration No. <u>43,963</u>
Richard B. LeBlanc	Registration No. <u>39,495</u>
Joseph D. Rossi	Registration No. <u>47,038</u>
George J. Awad	Registration No. <u>46,528</u>
Steven D. Maslowski	Registration No. <u>46,905</u>
S. Maurice Valla	Registration No. <u>43,966</u>
Emma R. Dailey	Registration No. <u>48,491</u>
Vincent J. Roccia	Registration No. <u>43,887</u>
Patrick J. Farley	Registration No. <u>42,524</u>
Stephen C. Timmins	Registration No. <u>48,481</u>
Erich M. Falke	Registration No. <u>49,049</u>
Jane E. Inglese	Registration No. <u>48,444</u>
Susan C. Murphy	Registration No. <u>46,221</u>
Raymond N. Scott, Jr.	Registration No. <u>48,666</u>
David L. Marcus	Registration No. <u>46,897</u>
Lawrence A. Aaronson	Registration No. <u>38,369</u>
John A. Harrelson	Registration No. <u>42,637</u>
Sharon Fenick	Registration No. <u>45,269</u>
Daniel D. Biesterveld	Registration No. <u>45,898</u>
Phillip A. Singer	Registration No. <u>40,176</u>
Richard D. Watkins	Registration No. <u>P50,993</u>
Wendy A. Choi	Registration No. <u>36,697</u>
Christine A. Goddard	Registration No. <u>46,731</u>
Andrew J. Hagerty	Registration No. <u>44,141</u>
Scott E. Scioli	Registration No. <u>47,930</u>
Felicity E. Groth	Registration No. <u>47,042</u>

#7

DOCKET NO. BTGI-0025

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Crisanto GUTIERREZ-ARMENTA and Elena RAMIREZ-PARRA

International Application No.: PCT/EP00/09325

International Filing Date: 25 September 2000 (25.09.2000)

For: WHEAT DP PROTEINS AND USES THEREOF

DECLARATION

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is for the following type of application:

(check one applicable item below)

- | | |
|---|--|
| <input type="checkbox"/> Original | <input type="checkbox"/> Divisional |
| <input type="checkbox"/> Continuation | <input checked="" type="checkbox"/> U.S. National Stage of PCT |
| <input type="checkbox"/> Continuation-in-Part | |

My residence, post office address and citizenship are as stated below next to my name; and I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

- | | |
|--|--|
| <input checked="" type="checkbox"/> Utility Patent | <input type="checkbox"/> Design Patent |
|--|--|

is sought on the invention, whose title appears above, the specification of which:

- | | |
|-------------------------------------|--|
| <input type="checkbox"/> | is attached hereto |
| <input type="checkbox"/> | was filed on _____, as U.S. Serial Number _____ |
| <input type="checkbox"/> | and was amended on _____ <i>(if applicable)</i> |
| <input checked="" type="checkbox"/> | as described and claimed in PCT International Application Number _____ |

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PATENT

PCT/EP00/09325, filed on 25 September 2000 and as amended
under PCT Article 19 on _____ and/or PCT Article 34 on
_____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

**DISCLOSURE AND/OR CLAIM FOR PRIORITY UNDER
35 U.S.C. §§ 119(a)-(d) OF FOREIGN APPLICATIONS
FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN)
OF THIS APPLICATION**

I hereby claim foreign priority benefits under Title 35, United States Code §§ 119(a)-(d) of any foreign applications for patent, inventor's certificate or PCT international application designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent, inventor's certificate or any PCT international application designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Country (or indicate if PCT)	Application Number	Date of Filing (day, month, year)	Priority Claimed Under 37 USC 119a-d
Spain	P9902127	24 September 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Spain	P9902474	11 November 1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No

**DISCLOSURE OF FOREIGN APPLICATION(S) IF ANY,
FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN)
PRIOR TO THE OF THIS APPLICATION**

Country (or indicate if PCT)	Application Number	Date of Filing (day, month, year)

DOCKET NO. BTGI-0025

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PATENT

**CLAIM FOR PRIORITY OF UNITED STATES APPLICATIONS
OR PCT APPLICATIONS FILED
IN THE UNITED STATES RECEIVING OFFICE
UNDER 35 U.S.C. §120**

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date Filed	Status Patented/Pending/Abandoned

**CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
UNDER 35 U.S.C. § 119(e)**

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date

DOCKET NO.: BTGI-0025

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PATENT

Send all future correspondence and direct all telephone calls to:

STRAHER, Michael P.
WOODCOCK WASHBURN LLP
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No.: (215) 568-3100
Facsimile No.: (215) 568-3439

- ☒ A Statement under 37 C.F.R. §3.73(b) and copies of relevant documents accompany this Power of Attorney.

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

Date: 05 / 06 / 2002By: Name: ROLF TARRACH SIEGELTitle: PRESIDENTE DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

DOCKET NO. BTGI-0025**- 4 -****PATENT**

Address all telephone calls, correspondence and maintenance fee correspondence to:

STRAHER, Michael P.
WOODCOCK WASHBURN LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone No.: **(215) 568-3100**
Facsimile No.: **(215) 568-3439**

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PATENT


I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first joint inventorCrisantoGutierrez-Armenta

(Given Name)

(Middle Initial or Name)

Family (or last name)

City/State of Actual Residence: Tres Cantos (Madrid) ESKMailing Address: Centro de Biologia Molecular "Severo Ochoa", Universidad Autonoma, Cantoblanco, E-28049 Madrid, SpainCountry of Citizenship: SpainInventor's signature: Date: June 17, 2002

DOCKET NO. BTGI-0025

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PATENT

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

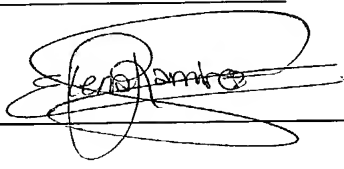
Full name of second joint inventor

Elena Ramirez-Parra
(Given Name) (Middle Initial or Name) Family (or last name)

City/State of Actual Residence: MADRID ESX

Mailing Address: Centro de Biologia Molecular "Severo Ochoa", Universidad Autonoma, Cantoblanco, E-28049 Madrid, Spain

Country of Citizenship: Spain

Inventor's signature: 

Date: June 19, 2002

SEQUENCE LISTING

<110> CONSEJO SUPERIOR DE INVESTICIONES CIENTIFICAS
GUTIERREZ-ARMENTA, CRISANTO
RAMIREZ-PARRA, ELENA

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Ser Val Pro Pro Leu Pro Glu Arg Gly Gly Asn Ala Val Gln Arg Lys
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ggg gct gtt gac ccg gat aaa gat agg aag gag aag gct gcg gca 196
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Ile Ala Leu Arg Val Ile Ala Lys Glu Lys Lys Glu Ile Arg Trp Met
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140 145 150 155

cgt aaa gaa ctc gtc aac aag att agg aac aag aag gca ctc ctc cag 532
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tatatatcct catgaaaact tgacctagtt tataggacag tctctcaggc ttgagaagat 942

tttaacctgc aaattttgtc tcctttttgt gcctagcagg ttattaggtc tcagatagat 1002

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cat gca aca aac gct aca caa agc agc aaa tca atc aat gtg gaa tat	1281
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Thr Gly Ser Gly Gly Ile Gly Ser Gly Gly Val Gly Gly Asp Ile Asp

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Val Thr Gly Asn Pro Leu Leu Thr Pro Val Ser Gly Lys Ala Val Lys		
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Cys Arg Tyr Asp Ser Ser Leu Gly Leu Leu Thr Lys Lys Phe Ile Asn		
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Glu Thr Leu Glu Val Gln Lys Arg Arg Ile Tyr Asp Ile Thr Asn Val		
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Trp Lys Gly Leu Asp Asp Ser Gly Val Glu Leu Asp Asn Gly Leu Ser		
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His Gly Thr Thr Leu Glu Val Pro Asp Pro Asp Glu Ala Gly Asp Tyr		
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Leu Gln Arg Arg Tyr Arg Ile Val Leu Arg Ser Thr Leu Gly Pro Ile		

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PCT/EP00/09325

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 Asp Lys Asp Arg Lys Lys Glu Lys Ala Ala Ala Pro Arg Ile Thr Gly
 1 5 10 15

Trp Gly Leu Arg Glu Tyr Ser Lys Ile Val Cys Glu Lys Val Glu Ala
 20 25 30

Lys Gly Arg Thr Thr Tyr Asn Glu Val Ala Asp Glu Ile Tyr Ser Glu
 35 40 45

Leu Lys Ser
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<210> 9
 <211> 168
 <212> DNA
 <213> Triticum monococcum

<220>
 <221> CDS
 <222> (1)..(168)

<400> 9
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 Met Ala His Ile Gly Gln Gly Phe Asp Glu Lys Asn Ile Arg Arg Arg
 1 5 10 15

gtg tat gat gct ttc aac gtt ctc att gca ctt cgt gtt att gca aaa 96
 Val Tyr Asp Ala Phe Asn Val Leu Ile Ala Leu Arg Val Ile Ala Lys
 20 25 30

gaa aaa aag gag ata cgg tgg atg ggc ctt tca aat tac aga tat gaa 144

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Glu Lys Lys Glu Ile Arg Trp Met Gly Leu Ser Asn Tyr Arg Tyr Glu
 35 40 45

aaa ata aag aag ctt gag gaa gtt 168
 Lys Ile Lys Lys Leu Glu Glu Val
 50 55

<210> 10

<211> 56

<212> PRT

<213> Triticum monococcum

<400> 10

Met Ala His Ile Gly Gln Gly Phe Asp Glu Lys Asn Ile Arg Arg Arg
 1 5 10 15

Val Tyr Asp Ala Phe Asn Val Leu Ile Ala Leu Arg Val Ile Ala Lys
 20 25 30

Glu Lys Lys Glu Ile Arg Trp Met Gly Leu Ser Asn Tyr Arg Tyr Glu
 35 40 45

Lys Ile Lys Lys Leu Glu Glu Val
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<210> 11

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<213> Triticum monococcum

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<222> (1)..(168)

<400> 11

cgt aaa gaa ctc gtc aac aag att agg aac aag aag gca ctc ctc cag 48
 Arg Lys Glu Leu Val Asn Lys Ile Arg Asn Lys Lys Ala Leu Leu Gln
 1 5 10 15

gaa atc gaa aaa cag ttt gat gat ctc caa aac atc aag tta cgt aac 96
 Glu Ile Glu Lys Gln Phe Asp Asp Leu Gln Asn Ile Lys Leu Arg Asn
 20 25 30

caa aca ctg gaa agc tca gca gag aat gtt aat ggc atc cgc ctt cca 144
 Gln Thr Leu Glu Ser Ser Ala Glu Asn Val Asn Gly Ile Arg Leu Pro

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35 40 45
 ttc gta ttg gtc aag aca tct agg 168
 Phe Val Leu Val Lys Thr Ser Arg
 50 55

<210> 12
 <211> 56
 <212> PRT
 <213> Triticum monococcum

<400> 12
 Arg Lys Glu Leu Val Asn Lys Ile Arg Asn Lys Lys Ala Leu Leu Gln
 1 5 10 15
 Glu Ile Glu Lys Gln Phe Asp Asp Leu Gln Asn Ile Lys Leu Arg Asn
 20 25 30
 Gln Thr Leu Glu Ser Ser Ala Glu Asn Val Asn Gly Ile Arg Leu Pro
 35 40 45
 Phe Val Leu Val Lys Thr Ser Arg
 50 55

<210> 13
 <211> 150
 <212> DNA
 <213> Triticum monococcum

<220>
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 <222> (1)..(150)

<400> 13
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 Lys Ala Arg Val Glu Ile Glu Ile Ser Asp Asp Ser Lys Phe Ala His
 1 5 10 15
 ttc gag ttc aat ggt gca cca ttc aca ttg cat gat gat ctc tca atc 96
 Phe Glu Phe Asn Gly Ala Pro Phe Thr Leu His Asp Asp Leu Ser Ile
 20 25 30
 ctt gag ggg gta agg cgt aac agc ata gga aga gct ggc cgc gcc acc 144
 Leu Glu Gly Val Arg Arg Asn Ser Ile Gly Arg Ala Gly Arg Ala Thr
 35 40 45

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ctt cac

150

Leu His

50

<210> 14

<211> 50

<212> PRT

<213> Triticum monococcum

<400> 14

Lys Ala Arg Val Glu Ile Glu Ile Ser Asp Asp Ser Lys Phe Ala His

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5

10

15

Phe Glu Phe Asn Gly Ala Pro Phe Thr Leu His Asp Asp Leu Ser Ile

20

25

30

Leu Glu Gly Val Arg Arg Asn Ser Ile Gly Arg Ala Gly Arg Ala Thr

35

40

45

Leu His

50

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<211> 25

<212> DNA

<213> Artificial Sequence

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<221> misc_binding

<222> (8)..(16)

<220>

<223> Description of Artificial Sequence:E2F canonical
binding site probe

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25

<210> 16

<211> 25

<212> DNA

<213> Artificial Sequence

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PCT/EP00/09325

<220>

<221> misc_binding

<222> (8)..(16)

<223> Mutant E2F Canonical binding site probe

<220>

<223> Description of Artificial Sequence:Mutant E2F
Canonical binding site probe

<400> 16

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25